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(54) Title: DETECTION USING DEGRADATION OF A TAGGED SEQUENCE

## (57) Abstract

Methods and compositions are provided for detecting DNA sequences, particularly single nucleotide polymorphisms, using a pair of nucleotide sequences, a primer and a SNP detection sequence, where the SNP detection sequence binds downstream from the primer to the target DNA in the direction of primer extension. The SNP detection sequence is characterized by having a nucleotide complementary to the SNP and adjacent nucleotides complementary to adjacent nucleotides in the target and an electrophoretic tag bonded to the 5'-nucleotide. The pair of sequences is combined with the target DNA under primer extension conditions, where the polymerase has 5'-3' exonuclease activity. When the SNP is present, the electrophoretic tag is released and can be detected by electrophoresis as indicative of the presence of the SNP in the target DNA. The sequence containing the SNP is exemplary of DNA sequences of interest generally.

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## DETECTION USING DEGRADATION OF A TAGGED SEQUENCE

### CROSS-REFERENCE TO RELATED APPLICATIONS

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This application is a continuation-in-part of application serial no. 09/303,029, filed April 30, 1999.

### INTRODUCTION

10

#### Field of the Invention

The field of this invention is multiplex sequence detection, exemplified by single nucleotide polymorphisms and gene expression, using electrophoretic separation.

#### 15 Background of the Invention

As the human genome is elucidated, there will be numerous opportunities for performing assays to determine the presence of specific sequences, distinguishing between alleles in homozygotes and heterozygotes, determining the presence of mutations, evaluating cellular expression patterns, etc. In many of these cases one will wish to determine in a single reaction, a number of different characteristics of the same sample. Also, there will be an interest in determining the presence of one or more pathogens, their antibiotic resistance genes, and the like.

In many assays, there will be an interest in determining the presence of specific sequences, whether genomic or cDNA. These sequences may be associated with particularly genes, regulatory sequences, repeats, multimeric regions, expression patterns, and the like

There is and will continue to be comparisons of the sequences of different individuals. It is believed that there will be about one polymorphism per 1,000 bases, so that one may anticipate that there will be an extensive number of differences between individuals. By single nucleotide polymorphism (snp's) is intended that there will be a prevalent nucleotide at the

site, with one or more of the remaining bases being present in substantially smaller percent of the population.

For the most part, the snp's will be in non-coding regions, primarily between genes, but will also be present in exons and introns. In addition, the great proportion of the snp's will not affect the phenotype of the individual, but will clearly affect the genotype. The snp's have a number of properties of interest. Since the snp's will be inherited, individual snp's and/or snp patterns may be related to genetic defects such as deletions, insertions and mutations involving one or more bases in genes. Rather than isolating and sequencing the target gene, it will be sufficient to identify the snp's involved.

In addition, the snp's may be used in forensic medicine to identify individuals. While other genetic markers are available, the large number of snp's and their extensive distribution in the chromosomes, make the snp's an attractive target. Also, by determining a plurality of snp's associated with a specific phenotype, one may use the snp pattern as an indication of the phenotype, rather than requiring a determination of the genes associated with the phenotype.

The need to determine many analytes or nucleic acid sequences (for example multiple pathogens or multiple genes or multiple genetic variants) in blood or other biological fluids has become increasingly apparent in many branches of medicine. The need to study differential expression of multiple genes to determine toxicologically-relevant outcomes or the need to screen transfused blood for viral contaminants with high sensitivity is clearly evident.

Thus most multi-analyte assays or assays which detect multiple nucleic acid sequences involve multiple steps, have poor sensitivity and poor dynamic range (2 to 100-fold differences in concentration of the analytes is determined) and some require sophisticated instrumentation.

Some of the known classical methods for multianalyte assays include the following:

- a. The use of two different radioisotope labels to distinguish two different analytes.
- b. The use of two or more different fluorescent labels to distinguish two or more analytes.
- c. The use of lanthanide chelates where both lifetime and wavelength are used to distinguish two or more analytes.

- d. The use of fluorescent and chemiluminescent labels to distinguish two or more analytes.
- e. The use of two different enzymes to distinguish two or more analytes.
- f. The use of enzyme and acridinium esters to distinguish two or more analytes.
- 5 g. Spatial resolution of different analytes for example arrays to identify and quantify multiple analytes.
- h. The use of acridinium ester labels where lifetime or dioxetanone formation is used to quantify two different viral targets.

10 Thus an assay that has higher sensitivity, large dynamic range ( $10^3$  to  $10^4$  – fold differences in target nucleic acids levels), and fewer and more stable reagents would increase the simplicity and reliability of multianalyte assays.

The need to identify and quantify a large number of bases or sequences distributed over potentially centimorgans of DNA offers a major challenge. Any method should be accurate, 15 reasonably economical in limiting the amount of reagents required and providing for a single assay, which allows for differentiation of the different SNP's or differentiation and quantitation of multiple genes.

#### Brief Description of the Related Art

20 Holland (Proc. Natl. Acad. Sci. USA (1991) 88:7276) discloses that the exonuclease activity of the thermostable enzyme Thermus aquaticus DNA polymerase in PCR amplification to generate specific detectable signal concomitantly with amplification.

The TAQMAN assay is discussed by Lee in Nucleic Acid Research (1993) 21:16 3761).

25 White (Trends Biotechnology (1996) 14(12):478-483) discusses the problems of multiplexing in the TAQMAN assay.

Marino, Electrophoresis (1996) 17:1499 describes low-stringency-sequence specific PCR (LSSP-PCR). A PCR amplified sequence is subjected to single primer amplification under conditions of low stringency to produce a range of different length amplicons. Different

patterns are obtained when there are differences in sequence. The patterns are unique to an individual and of possible value for identity testing.

Single strand conformational polymorphism (SSCP) yields similar results. In this method the PCR amplified DNA is denatured and sequence dependent conformations of the single strands are detected by their differing rates of migration during gel electrophoresis. As with LSSP-PCR above, different patterns are obtained that signal differences in sequence. However, neither LSSP-PCR nor SSCP gives specific sequence information and both depend on the questionable assumption that any base that is changed in a sequence will give rise to a conformational change that can be detected. Pastinen, Clin. Chem. (1996) 42:1391 amplifies the target DNA and immobilizes the amplicons. Multiple primers are then allowed to hybridize to sites 3' and contiguous to an SNP site of interest. Each primer has a different size that serves as a code. The hybridized primers are extended by one base using a fluorescently labeled dideoxynucleoside triphosphate. The size of each of the fluorescent products that is produced, determined by gel electrophoresis, indicates the sequence and, thus, the location of the SNP. The identity of the base at the SNP site is defined by the triphosphate that is used. A similar approach is taken by Haff, Nucleic Acids Res. (1997) 25:3749 except that the sizing is carried out by mass spectroscopy and thus avoids the need for a label. However, both methods have the serious limitation that screening for a large number of sites will require large, very pure primers that can have troublesome secondary structures and be very expensive to synthesize.

Hacia, Nat. Genet. (1996) 14:441 uses a high density array of oligonucleotides. Labeled DNA samples were allowed to bind to 96,600 20-base oligonucleotides and the binding patterns produced from different individuals were compared. The method is attractive in that SNP's can be directly identified but the cost of the arrays is high.

Fan (1997, October 6-8, IBC, Annapolis MD) has reported results of a large scale screening of human sequence-tagged sites. The accuracy of single nucleotide polymorphism screening was determined by conventional ABI resequencing.

Allele specific oligonucleotide hybridization along with mass spectroscopy has been discussed by Ross in Anal. Chem. (1997) 69:4197.

Holland, et al., PNAS USA (1991) 88, 7276-7280, describes use of DNA polymerase 5'-3' exonuclease activity for detection of PCR products.

## 5

SUMMARY OF THE INVENTION

Multiplexed sequence detection, exemplified by snp's and gene expression analysis, is provided by employing a combination of a primer and labeled detector sequence probe in the presence of primer extension reagents, where the polymerase includes 5'-3' exonuclease 10 activity. The labeled detector sequence prob. in the case of snps has at least one nucleotide, which is substituted with an electrophoretic tag. One combines the target nucleic acid, which will usually have been processed, with the primer extension reagents and at least one pair for each nucleic acid sequence of interest under conditions for primer extension. After sufficient time for primer extension to occur with degradation of detector sequences bound to target 15 nucleic acid, the electrophoretic tag labeled nucleotides are separated and detected. By having a different electrophoretic tag for each nucleic acid sequence of interest, having a different electrophoretic mobility, which may require further treatment depending on the total number of snp's or target sequences to be detected, one can readily determine the snp's or measure multiple sequences, which are present in a sample..

20 Electrophoretic tags are small molecules (Molecular weight of 150 to 10,000), usually other than oligonucleotides, which can be used in any measurement technique that permits identification by mass, e.g. mass spectrometry, and or mass/charge ratio, as in mobility in electrophoresis . Simple variations in mass and/or mobility of the electrophoretic tag leads to generation of a library of electrophoretic tags, that can then be used to detect multiple snp's or 25 multiple target sequences. The electrophoretic tags are easily and rapidly separated in free solution without the need for a polymeric separation media. Quantitation is achieved using internal controls. Enhanced separation of the electrophoretic tags in electrophoresis is achieved by modifying the tags with positively charged moieties.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A, B and C depict the specific sequences of the snp detection sequences for the two alleles, the optical characteristics of the fluorescent dyes, and the cleaved fragments from 5 the snp detection sequences, respectively;

Figs. 2A and B depict the CE separation of the reaction products of Allele 1 after 0 and 40 cycles. CE instrument: Beckman P/ACE/ 5000 with LIF detection. BGE: 2.5% LLD 30, 7M urea, 1xTBE. Capillary: 100 $\mu$ m i.d., 375 $\mu$ m o.d., Lc=27cm, Ld=6.9cm. Detection:  $\lambda_{ex}=488nm$ ,  $\lambda_{em}=520nm$ . Injection: 5s at 2.0kV. Field strength: 100V/cm at rt. Peaks: P= 10 unreacted snp detection sequence, P' = snp detection sequence product

Figs. 3A and B depict the CE separation of the reaction products of Allele 1 after 0 and 40 cycles. Experimental conditions are the same as Fig. 2, except for BGE composition; 2.% LDD30, 1 x TBE;

Fig. 4 is a graph of the CE separation of a 1:1 mixture of the 40 cycles products of 15 Alleles 1 and 2, with experimental conditions as described for Fig. 2; and

Fig. 5 is a graph of the CE separation of a 1:10 mixture of the 40 cycles products of Alleles 1 and 2, with experimental conditions as described for Fig. 2.

Fig. 6 is an electropherogram of electrophoretic tags which involved a separation involving a 1000-fold difference in concentration.

20 Figs. 7a, 7b are the electropherograms of the analysis of 5 snps of the cystic fibrosis genes using multiplexed PCR and the subject E-TAG probes. Fig. 7c is the electropherogram of the analysis of single snps and triple x snps for the cystic fibrosis genes using multiplexed PCR and the subject E-TAG probes along with an agarose gel separation of the triple x PCR.

Fig. 8 is an electropherogram of a separation of 9 negatively charged E-TAG probes.

25 Figs. 9A and 9B are electropherograms of probes employing a penultimate thiophosphate linkage in the E-TAG probes to discourage cleavage after the first phosphate linkage.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods and compositions are provided for improved analysis of complex nucleic acid mixtures, where one is interested in the simultaneous identification of a plurality of entities,

5 such as sequences, snps, alleles, mutations, etc. The methodology involves employing detectable tags that can be differentiated by mass, usually mobility, where the tags are generated by cleavage from a nucleic acid sequence of the probe. Improvements in the detectable tags provide for having a positively multicharged moiety joined to the electrophoretic-tagged probe, employing a blocking linkage between at least the second and

10 third nucleotides to inhibit cleavage at this or subsequent sites, and using control sequences for quantitation. The subject invention may be used for a variety of nucleic acid analyses.

However, since SNP detection is, for the most part, the most stringent in its requirements, most of the description will be directed toward the multiplexed detection of SNPs. For other nucleic acid analyses, frequently the protocols will be substantially the same, although in some 15 instances somewhat different protocols will be employed for SNPs, because of the greater demands SNPs make on fidelity.

One, usually a plurality, of SNP's, is simultaneously determined by combining target DNA with one or a plurality, respectively, of reagent pairs under conditions of primer extension. Each pair of reagents includes a primer which binds to target DNA and a SNP 20 detection sequence, normally labeled, which binds to the site of the SNP and has an electrophoretic tag, usually at its 5'-end and the base complementary to the SNP, usually at other than a terminus of the SNP detection sequence. The conditions of primer extension employ a polymerase having 5'-3' exonuclease activity, dNTP's and auxiliary reagents to permit efficient primer extension. The primer extension is performed, whereby detector 25 sequences bound to the target DNA are degraded with release of the electrophoretic tag. By having each SNP associated with its own electrophoretic tag, one can determine the SNP's, which are present in the target DNA for which pairs of reagents have been provided.

The pairs of reagents are DNA sequences, which are related to a SNP site. The primer binds to the target DNA upstream from the SNP site in the direction of extension. The labeled 30 detector sequence binds downstream from the primer in the direction of extension and binds to

a sequence, which includes the SNP. The primer sequence will usually be at least about 12 bases long, more usually at least 18 bases long and usually fewer than 100 bases, and more usually fewer than 60 bases. The primer will be chosen to bind substantially uniquely to a target sequence under the conditions of primer extension, so that the sequence will normally  
5 be one that is conserved or the primer is long enough to bind in the presence of a few mismatches, usually fewer than about 10 number % mismatches. By knowing the sequence, which is upstream from the SNP of interest, one may select a sequence, which has a high G-C ratio, so as to have a high binding affinity for the target sequence. In addition, the primer should bind reasonably close to the SNP, usually not more than about 200 bases away, more  
10 usually not more than about 100 bases away, and preferably within about 50 bases. Since the farther away the primer is from the SNP, the greater amount of dNTP's, which will be expended, there will usually be no advantage in having a significant distance between the primer and the SNP detection sequence. Generally, the primer will be at least about 5 bases away from the SNP.

15 The number of reagent pairs may be varied widely, from a single pair to two or more pairs, usually at least about 5 pairs, more usually at least about 9 pairs and may be 20 pairs or more. By virtue of the use of different E-TAGs, which have different mobilities and are readily resolvable under conventional capillary electrophoretic conditions, the subject pairs may be used to perform multiplexed operations in a single vessel, where a family of SNPs may  
20 be identified. Usually, the total number of different reagent pairs or different target sequences in a single determination will be under 200, more usually under 100 and in many cases will not exceed 50

In one SNP determination protocol, the primer terminates prior to the complementary base of the SNP. This protocol is referred to as "Invader" technology and is described in U.S.  
25 Patent no. 6,001,567. The protocol involves providing: (a) (i) a cleavage means, which is normally an enzyme, referred to as a cleavase, that recognizes a triplex consisting of the target sequence, a primer which binds to the target sequence and terminates at the SNP sequence and a labeled probe that binds immediately adjacent to the primer and is displaced from the target at the SNP position, when a SNP is present; the cleavase clips the labeled probe at the site of  
30 displacement, releasing the label; ii) a source of target nucleic acid, the target nucleic acid

having a first region, a second region and a third region, wherein the first region is downstream from the second region and the second region is contiguous to and downstream from the third region; and (iii) first and second oligonucleotides having 3' and 5' portions, wherein the 3' portion of the first oligonucleotide contains a sequence complementary to the 5 3rd region of the target nucleic acid and the 5' portion of the first oligonucleotide and the 3' portion of the second oligonucleotide each contain sequence fully complementary to the second region of the target nucleic acid, and the 5' portion of the second oligonucleotide contains sequence complementary to the first region of said target nucleic acid; (b) mixing, in any order, the cleavage means, the target nucleic acid, and the first and second oligonucleotides 10 under hybridization conditions that at least the 3' portion of the first oligonucleotide is annealed to the target nucleic acid and at least the 5' portion of the second oligonucleotide is annealed to any target nucleic acid to form a cleavage structure, where the combined melting temperature of the complementary regions within the 5' and 3' portions of the first oligonucleotide when annealed to the target nucleic acid is greater than the melting 15 temperature of the 3' portion of the first oligonucleotide and cleavage of the cleavage structure occurs to generate labeled products; and (c) detecting the labeled cleavage products.

Thus, in an Invader assay attachment of an electrophoretic tag to the 5' end of the detector sequence results in the formation of electrophoretic tag labeled nucleotide when target sequence is present. The electrophoretic tag labeled nucleotide is separated and 20 detected. By having a different electrophoretic tag for each nucleic acid sequence of interest, having a different electrophoretic mobility, which may require further treatment depending on the total number of snp's or target sequences to be detected, one can readily determine the snp's or measure multiple sequences, which are present in a sample.

In another snp detection protocol, an alternative method of cleavage is used and various 25 detectable tags may be employed, the most common using a fluorescent label. The difference in protocol between a fluorescent label and another type of label, such as an electrochemical label, is the method of detection. Otherwise, the protocols will be substantially the same. The tagged snp detection sequence will be chosen to bind to the target sequence comprising the snp. The length of the snp detector sequence is in part related to the length and binding 30 affinity of the primer. The two sequences act together to ensure that the pair of reagents bind

to the proper target sequence. The greater the fidelity of binding of one member of the pair, the less fidelity that is required for the other member of the pair. Since the observed signal will be dependent upon both members of the pair being present, each member serves as a check on the other member for production of the signal. However, since except for the cost, 5 it is relatively easy to make reasonably long oligonucleotides, usually both members of the pair will provide for unique binding to their respective target sequences. Therefore, the length of the snp detector sequence will come within the parameters indicated for the primer, but the total number of bases for the two pair members will usually be at least 36, more usually at least about 40.

10 Each snp detection sequence will have at least one nucleotide modified with an electrophoretic tag, which is labeled, which is fluorescent or can be subsequently made fluorescent, or can be detected electrochemically or by other convenient detection methodologies. Usually, the modified nucleotide will be at the 5'-end of the sequence, but the modified nucleotide may be anywhere in the sequence, particularly where there is a single 15 nuclease susceptible linkage in the detection sequence. Since the determination is based on the at least partial degradation of the snp detector sequence, having the modified nucleotide at the end ensures that if degradation occurs, the electrophoretic tag will be released. Since nucleases may clip at other than the terminal phosphate link, it is desirable to prevent cleavage at other than the terminal phosphate link. In this way one avoids the confusion of having the 20 same electrophoretic tag joined to different numbers of nucleotides after cleavage. Cleavage at the terminal phosphate can be relatively assured by using a linker at the penultimate nucleoside, which is not cleaved by the nuclease, more particularly having only the ultimate linkage susceptible to hydrolysis by a nuclease. For example, one may use a thiophosphate, phosphinate, phosphoramidate, or a linker other than a phosphorous acid derivative, such as 25 an amide, boronate, or the like. The particular hydrolase resistive linker will be primarily one of synthetic convenience, so long as degradation of the binding affinity is not sacrificed. If desired all of the linkers other than the ultimate linker may be resistant to nuclease hydrolysis. The snp detection sequence may be further modified to improve separation and detection of the electrophoretic tags. By virtue of the difference in mobility of the electrophoretic tags, the 30 snp detection sequences will also have different mobilities. Furthermore, these molecules

will be present in much larger amounts than the released electrophoretic tags, so that they may obscure detection of the released electrophoretic tags. Also, it is desirable to have negatively charged SNP detection sequence molecules, since they provide for higher enzymatic activity and decrease capillary wall interaction. Therefore, by providing that the intact SNP detection sequence molecule can be modified with a positively charged moiety, but not the released electrophoretic tag, one can change the electrostatic nature of the SNP detection sequence molecules during the separation. By providing for a ligand on the SNP detection sequence molecule to which a positively charged molecule can bind, one need only add the positively charged molecule to change the electrostatic nature of the SNP detection sequence molecule.

5 Conveniently, one will usually have a ligand of under about 1kDa. This may be exemplified by the use of biotin as the ligand and avidin, which is highly positively charged, as the receptor/positively charged molecule. Instead of biotin/avidin, one may have other pairs, where the receptor, e.g. antibody, is naturally positively charged or is made so by conjugation with one or more positively charged entities, such as arginine, lysine or histidine, ammonium,

10 etc. The presence of the positively charged moiety has many advantages in substantially removing the SNP detection sequence molecules from the electropherogram. In carrying out the process, the positively charged moiety is added at or after the completion of the digestion.

15

Another way of removing nonspecific degradation products of detection sequence is to attach a ligand, exemplified by biotin, to the penultimate nucleoside. After the 5' nuclease assay, a receptor for the ligand, for biotin exemplified by streptavidin (hereafter "avidin") is added to the assay mixture. Other receptors include natural or synthetic receptors, such as immunoglobulins, lectins, enzymes, etc. Desirably, the receptor is positively charged, naturally as in the case of avidin, or is made so, by the addition of a positively charged moiety or moieties, such as ammonium groups, basic amino acids, etc. Avidin binds to the biotin attached to the detection probe and its degradation products. Avidin is positively charged, while the cleaved electrophoretic tag is negatively charged. Thus the separation of the cleaved electrophoretic tag from, not only uncleaved probe, but also its degradation products, is easily achieved by using conventional separation methods. Alternatively, the receptor may be bound to a solid support or high molecular weight macromolecule, such as a vessel wall, particles,

20

25

30 e.g. magnetic particles, cellulose, agarose, etc., and separated by physical separation or

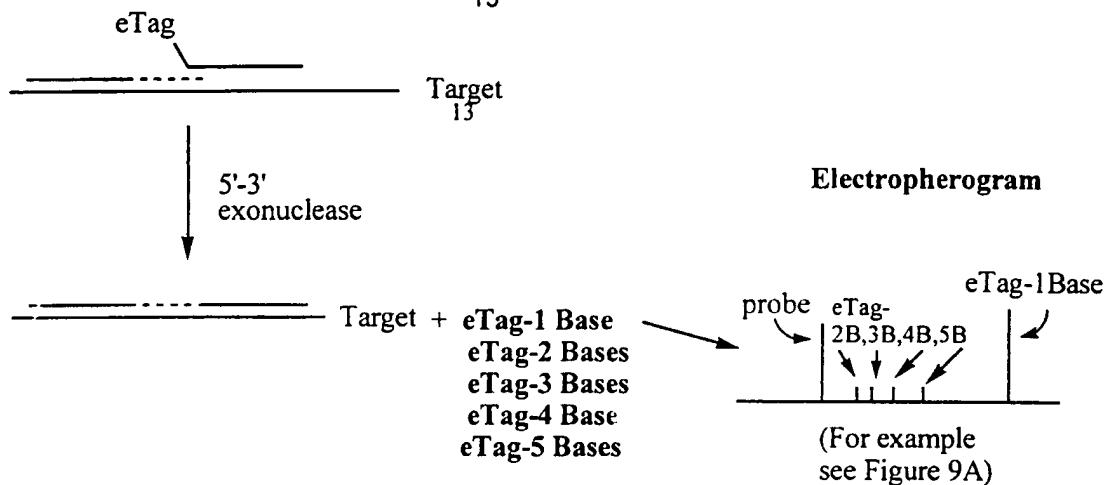
centrifugation, dialysis, etc. This method further enhances the specificity of the assay and allows for a higher degree of multiplexing.

While the ligand may be present at a position other than the penultimate position and one may make the ultimate linkage nuclease resistant, so that cleavage is directed to the 5 penultimate linkage, this will not be as efficient as having cleavage at the ultimate linkage. The efficiency would be even worse where the ligand is at a more distant nucleotide from the E-TAG. Therefore, while such protocols are feasible, and may be used, they will not be preferred.

Where the electrophoretic tags ("E-TAGs") are used for mass detection, as with mass 10 spectrometry, the E-TAGs need not be charged but merely differ in mass, since a charge will be imparted to the E-TAG by the mass spectrometer. The use of the subject method of freeing the E-TAGs of an extended number of nucleotides as a result of cleavage at other than the ultimate linkage finds advantage in any separation technique, where the additional peaks from the series of cleavage products will interfere with analyzing the data, as in mass spectrometry.

15 The above three methods are generally applicable not only to generating a single electrophoretic tag per sequence detected but also to generation of a single oligonucleotide fragment for fragment separation and identification by electrophoresis or by mass spectra as it is essential to get one fragment per sequence detected. For purpose of explanation, these methods are illustrated below.

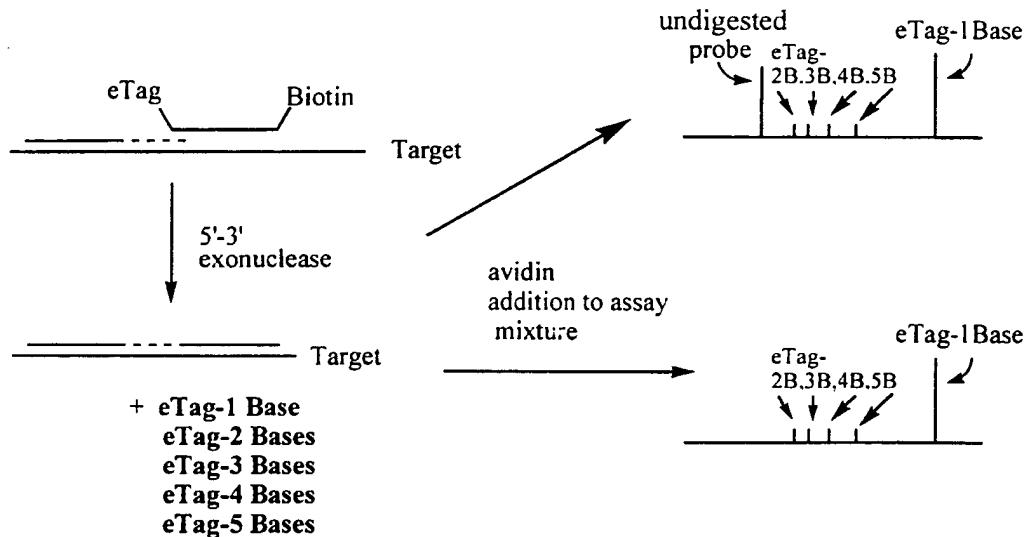
13



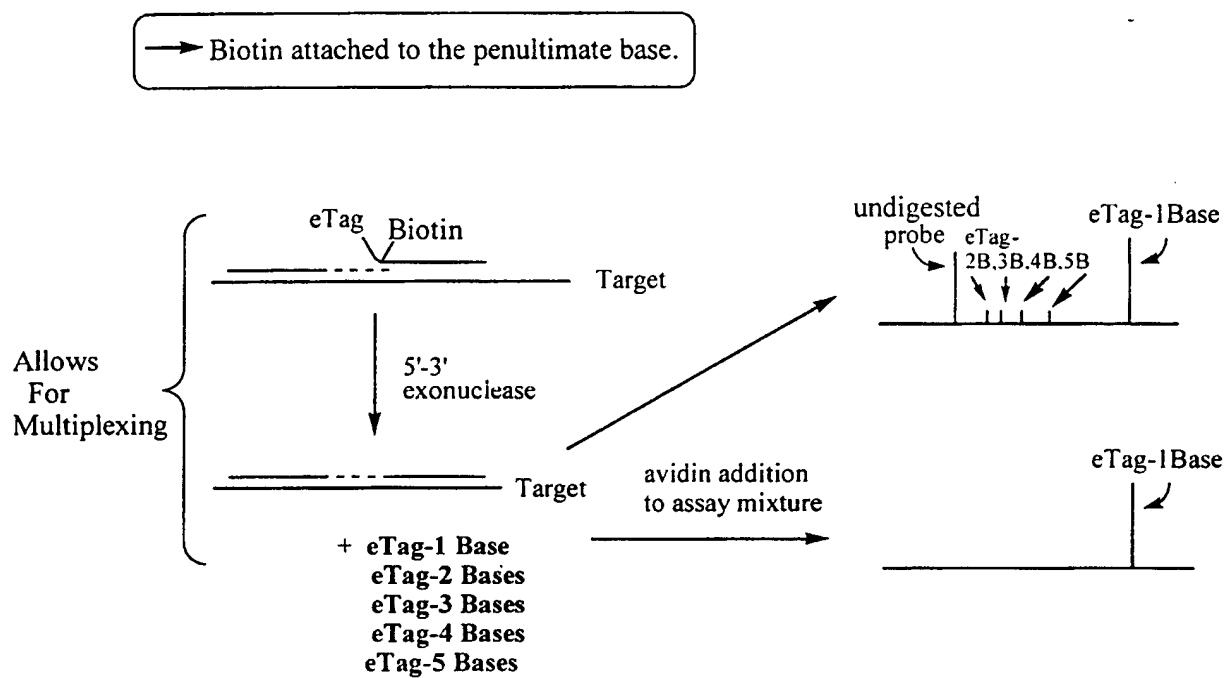
→ It is imperative that only one eTag be generated per target for maximum multiplexing capabilities.

2. Depurination results in degradation of signal probes.
3. Signal probe is also degraded by the enzyme (5'-3' nuclease activity).

### Approach I.

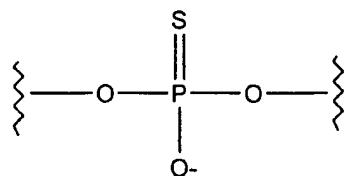


## Approach II.



## Approach III.

→ Nuclease resistant modifications to backbone.  
Ex. Phosphorothioates.



The complementary base to the SNP may be anywhere in the detector sequence, desirably at other than the terminal nucleoside to enhance the fidelity of binding. The SNP detector sequence will be designed to include adjacent nucleotides, which provide the desired affinity for the hybridization conditions. The SNP detection sequence may be synthesized by any convenient means, such as described in Matthews, et al., Anal Biochem. (1988) 169:1 –

25; Keller, et al., "DNA Probes," 2<sup>nd</sup> edition (1993) Stockton Press, New York, NY; and Wetmur, Critical Reviews in Biochemistry and Molecular Biology (1991) 26:227 - 259.

The electrophoretic tag will be one, which is labeled with a directly detectable label or can be made so by functionalization. The electrophoretic tags will be differentiated by their 5 electrophoretic mobility, usually their mass/charge ratio, to provide different mobilities for each electrophoretic tag. Although in some instances the electrophoretic tags may have identical mass/charge ratios, such as oligonucleotides but differ in size or shape and therefore exhibit different electrophoretic mobilities under appropriate conditions. Therefore, the tags will be amenable to electrophoretic separation and detection, although other methods of 10 differentiating the tags may also find use. The electrophoretic tag may be joined to any convenient site on the nucleotide, without interfering with the synthesis of the snp detector sequence and without interfering with the hydrolysis of the phosphate linkage to release the electrophoretic tag. Thus, the tag may be bound to a site on the base, either an annular carbon atom or a hydroxyl or amino substituent. The electrophoretic tag may be linked by a stable 15 bond or one, which may be cleavable, thermally, photolytically or chemically. There is an interest in cleaving the electrophoretic tag from the nucleotide where the snp detector sequence results in significant cleavage at other than the 5'-phosphate link, resulting in di- and higher oligonucleotides and this family of products interferes with the separation and detection of the electrophoretic tags. However, rather than requiring an additional step in the identification of 20 the tags by releasing them from the base to which they are attached, one can modify the snp detection sequence to minimize obtaining cleavage at other than the ultimate linker.

If present, the nature of the releasable link may be varied widely. Numerous linkages are available, which are thermally, photolytically or chemically labile. See, for example, U.S. Patent no. 5,721,099. Where detachment of the product is desired, there are numerous 25 functionalities and reactants, which may be used. Conveniently, ethers may be used, where substituted benzyl ether or derivatives thereof, e.g. benzhydryl ether, indanyl ether, etc. may be cleaved by acidic or mild reductive conditions. Alternatively, one may employ beta-elimination, where a mild base may serve to release the product. Acetals, including the thio analogs thereof, may be employed, where mild acid, particularly in the presence of a 30 capturing carbonyl compound, may serve. By combining formaldehyde, HCl and an alcohol

moiety, an  $\alpha$ -chloroether is formed. This may then be coupled with an hydroxy functionality to form the acetal. Various photolabile linkages may be employed, such as o-nitrobenzyl, 7-nitroindanyl, 2-nitrobenzhydryl ethers or esters, etc.

For a list of cleavable linkages, see, for example, Greene and Wuts, Protective Groups in Organic Synthesis, 2<sup>nd</sup> ed. Wiley, 1991. The versatility of the various systems that have been developed allows for broad variation in the conditions for attachment of the electrophoretic tag entities.

Various functionalities for cleavage are illustrated by: silyl groups being cleaved with fluoride, oxidation, acid, bromine or chlorine; o-nitrobenzyl with light; catechols with cerium salts; olefins with ozone, permanganate or osmium tetroxide; furans with oxygen or bromine in methanol; tertiary alcohols with acid; ketals and acetals with acid;  $\alpha$ - and  $\beta$ -substituted ethers and esters with base, where the substituent is an electron withdrawing group, e.g., sulfone, sulfoxide, ketone, etc., and the like

The electrophoretic tags will have a linker, which provides the linkage between the base and the detectable label molecule, usually a fluorescer, or a functionality which may be used for linking to a detectable label molecule. By having different functionalities, which may be individually bonded to a detectable label molecule, one enhances the opportunity for diversity of the electrophoretic tags. Using different fluorescers for joining to the different functionalities, the different fluorescers can provide differences in light emission and mass/charge ratios for the electrophoretic tags.

The linkers may be oligomers, where the monomers may differ as to mass and charge. For convenience and economy, monomers will generally be commercially available, but if desired, they may be originally synthesized. Monomers which are commercially available and readily lend themselves to oligomerization include amino acids, both natural and synthetic, monosaccharides, both natural and synthetic, while other monomers include hydroxyacids, where the acids may be organic or inorganic, e.g. carboxylic, phosphoric, boric, sulfonic, etc., and amino acids, where the acid is inorganic, and the like. In some instances, nucleotides, natural or synthetic, may find use. The monomers may be neutral, negatively charged or positively charged. Normally, the charges of the monomers in the linkers will be the same, so that in referring to the mass/charge ratio, it will be related to the same charge.

Where the label has a different charge from the linker, this will be treated as if the number of charges is reduced by the number of charges on the linker. For natural amino acids, the positive charges may be obtained from lysine, arginine and histidine, while the negative charges may be obtained from aspartic and glutamic acid. For nucleotides, the charges will be obtained from the phosphate and any substituents that may be present or introduced onto the base. For sugars, sialic acid and uronic acids of the various sugars, or substituted sugars may be employed.

For the most part, the linker may be a bond, where the label is directly bonded to the nucleoside, or a link of from 1 to 500 or more, usually 1 to 300 atoms, more usually 2 to 100 atoms in the chain. The total number of atoms in the chain will depend to a substantial degree on the diversity required to recognize all the SNP's to be determined. The chain of the linker for the most part will be comprised of carbon, nitrogen, oxygen, phosphorous, boron, and sulfur. Various substituents may be present on the linker, which may be naturally present as part of the naturally occurring monomer or introduced by synthesis. Functionalities which may be present in the chain include amides, phosphate esters, ethers, esters, thioethers, disulfides, borate esters, sulfate esters, etc. The side chains include amines, ammonium salts, hydroxyl groups, including phenolic groups, carboxyl groups, esters, amides, phosphates, heterocycles, particularly nitrogen heterocycles, such as the nucleoside bases and the amino acid side chains, such as imidazole and quinoline, thioethers, thiols, or other groups of interest to change the mobility of the electrophoretic tag. The linker may be a homooligomer or a heterooligomer, having different monomers of the same or different chemical characteristics, e.g., nucleotides and amino acids.

The linker may be joined in any convenient manner to the base of the nucleoside. Various functionalities which may be used include alkylamine, amidine, thioamide, ether, urea, thiourea, guanidine, azo, thioether and carboxylate, sulfonate, and phosphate esters, amides and thioesters.

Besides the nature of the linker, as already indicated, diversity can be achieved by the chemical and optical characteristics of the label, the use of energy transfer complexes, variation in the chemical nature of the linker, which affects mobility, such as folding, interaction with the solvent and ions in the solvent, and the like. As already suggested, the

linker will usually be an oligomer, where the linker may be synthesized on a support or produced by cloning or expression in an appropriate host. Conveniently, polypeptides can be produced where there is only one cysteine or serine/threonine/tyrosine, aspartic/glutamic acid, or lysine/arginine/histidine, other than an end group, so that there is a unique functionality, 5 which may be differentially functionalized. By using protective groups, one can distinguish a side chain functionality from a terminal amino acid functionality. Also, by appropriate design, one may provide for preferential reaction between the same functionalities present at different sites on the linking group. Whether one uses synthesis or cloning for preparation of oligopeptides, will to a substantial degree depend on the length of the linker.

10 The electrophoretic tag, which is detected, will comprise the linker, generally a label, and optionally the nucleotide of the snp detection sequence. Generally, the electrophoretic tag will have a charge/mass ratio in the range of about -0.0001 to 1, usually in the range of about -0.001 to about 0.5. Mobility is  $q/M^{2/3}$ , where q is the charge on the molecule and M is the mass of the molecule. Desirably, the difference in mobility under the conditions of the 15 determination between the closest electrophoretic labels will be at least about 0.05, more usually at least about 0.1

If desired, the snp detection sequence may have a combination of a quencher and a fluorescer. In this instance the fluorescer would be in proximity to the nucleoside to which the linker is bonded, as well as the quencher, so that in the primer extension mixture, fluorescence 20 from fluorescer bound to the snp detection sequence would be quenched. As the reaction proceeds and fluorescer is released from the snp detection sequence and, therefore, removed from the quencher, it would then be capable of fluorescence. By monitoring the primer extension mixture for fluorescence, one would be able to determine when there would probably be a sufficient amount of individual electrophoretic tags to provide a detectable signal 25 for analysis. In this way, one could save time and reagent by terminating the primer extension reaction at the appropriate time. There are many quenchers that are not fluorescers, so as to minimize fluorescent background from the snp detection sequence. Alternatively, one could take small aliquots and monitor the reaction for observable electrophoretic tags.

The snp detection sequence may be further modified to improve separation and 30 detection of the electrophoretic tags. By virtue of the difference in mobility of the

electrophoretic tags, the SNP detection sequences will also have different mobilities. Furthermore, these molecules will be present in much larger amounts than the released electrophoretic tags, so that they may obscure detection of the released electrophoretic tags. Also, it is desirable to have negatively charged SNP detection sequence molecules, since they provide for higher enzymatic activity and decrease capillary wall interaction. Therefore, by providing that the intact SNP detection sequence molecule can be modified with a positively charged moiety, but not the released electrophoretic tag, one can change the electrostatic nature of the SNP detection sequence molecules during the separation. By providing for a ligand on the SNP detection sequence molecule to which a positively charged molecule can bind, one need only add the positively charged molecule to change the electrostatic nature of the SNP detection sequence molecule. Conveniently, one will usually have a ligand of under about 1kDa. This may be exemplified by the use of biotin as the ligand and avidin, which is highly positively charged, as the receptor/positively charged molecule. Instead of biotin/avidin, one may have other pairs, where the receptor, e.g. antibody, is naturally positively charged or is made so by conjugation with one or more positively charged entities, such as arginine, lysine or histidine, ammonium, etc. The presence of the positively charged moiety has many advantages in substantially removing the SNP detection sequence molecules from the electropherogram. In carrying out the process, the positively charged moiety is added at or after the completion of the digestion.

The extension reaction is performed by bringing together the necessary combination of reagents and subjecting the mixture to conditions for carrying out the desired primer extension. Such conditions depend on the nature of the extension, e.g., PCR, single primer amplification, LCR, NASBA, 3SR and so forth, where the enzyme which is used for the extension has 5'-3' nuclease activity. The extension reaction may be carried out as to both strands or as to only a single strand. Where pairs of primer and SNP detection sequence are used for both strands, conveniently, the electrophoretic tag will be the same, but the bases will be different. In this situation, one may wish to have a cleavable linkage to the base, so that for the same SNP, one would obtain the same electrophoretic tag. Alternatively, if the number of SNP's to be determined is not too high, one could use different electrophoretic tags for each of the strands. Usually, the reaction will be carried out by using amplifying conditions, so as

to provide an amplified signal for each snp. Amplification conditions normally employ thermal cycling, where after the primer extension and release of electrophoretic tags associated with snps' which are present, the mixture is heated to denature the double-stranded DNA, cooled, where the primer and snp detection sequence can rehybridize and the extension 5 repeated.

Depending on the protocol, the electrophoretic tags or E-TAG, will be separated from a portion or substantially all of the detection sequence, usually retaining not more than about 3 nucleotides, more usually not more than about 2 nucleotides and preferably from 0 to 1 nucleotide. By having a cleavable linker between the E-TAG and the detection sequence, the 10 E-TAG may be freed of all the nucleotides. By having a nuclease resistant penultimate link, a single nucleotide may be bonded to the E-TAG.

Reagents for conducting the primer extension are substantially the same reaction materials for carrying out an amplification, such as an amplification indicated above. The nature and amounts of these reagents are dependent on the type of amplification conducted. In 15 addition to oligonucleotide primers the reagents also comprise nucleoside triphosphates and a nucleotide polymerase having 5'-3' nuclease activity.

The nucleoside triphosphates employed as reagents in an amplification reaction include deoxyribonucleoside triphosphates such as the four common deoxyribonucleoside triphosphates dATP, dCTP, dGTP and dTTP. The term "nucleoside triphosphates" also 20 includes derivatives and analogs thereof, which are exemplified by those derivatives that are recognized and polymerized in a similar manner to the underivatized nucleoside triphosphates.

The nucleotide polymerase employed is a catalyst, usually an enzyme, for forming an extension of an oligonucleotide primer along a polynucleotide such as a DNA template, where the extension is complementary thereto. The nucleotide polymerase is a template dependent 25 polynucleotide polymerase and utilizes nucleoside triphosphates as building blocks for extending the 3'-end of a polynucleotide to provide a sequence complementary with the polynucleotide template. Usually, the catalysts are enzymes, such as DNA polymerases, for example, prokaryotic DNA polymerase (I, II, or III), T4 DNA polymerase, T7 DNA polymerase, Vent DNA polymerase, *Pfu* DNA polymerase, *Taq* DNA polymerase, and the 30 like. Polymerase enzymes may be derived from any source, such as eukaryotic or prokaryotic

cells, bacteria such as *E. coli*, plants, animals, virus, thermophilic bacteria, genetically modified enzymes, and so forth.

The conditions for the various amplification procedures are well known to those skilled in the art. In a number of amplification procedures, thermal cycling conditions as discussed above are employed to amplify the polynucleotides. The combination of reagents is subjected to conditions under which the oligonucleotide primer hybridizes to the priming sequence of, and is extended along, the corresponding polynucleotide. The exact temperatures can be varied depending on the salt concentration, pH, solvents used, length of and composition of the target polynucleotide sequence and the oligonucleotide primers.

Thermal cycling conditions are employed for conducting an amplification involving temperature or thermal cycling and primer extension, such as in PCR or single primer amplification, and the like. The pH and the temperature are selected so as to cause, either simultaneously or sequentially, dissociation of any internally hybridized sequences, hybridization or annealing of the oligonucleotide primer and snp detection sequence with the target polynucleotide sequence, extension of the primer, release of the electrophoretic tag from snp detection sequence bound to the target polynucleotide sequence and dissociation of the extended primer. This usually involves cycling the reaction medium among two or more temperatures. In conducting such a method, the medium is cycled between two to three temperatures. The temperatures for thermal cycling generally range from about 50°C to 100°C, more usually, from about 60°C to 95°C. Relatively low temperatures of from about 30°C to about 65°C can be employed for the extension steps, while denaturation and hybridization can be carried out at a temperature of from about 50°C to about 105°C. The reaction medium is initially at about 20°C to about 45°C, preferably, about 25°C to about 35°C. Relatively low temperatures of from about 50°C to about 80°C, preferably, 50°C to about 60°C, are employed for the hybridization or annealing steps, while denaturation is carried out at a temperature of from about 80°C to about 100°C, preferably, 90°C to about 95°C, and extension is carried out at a temperature of from about 70°C to about 80°C, usually about 72°C to about 74°C. The duration of each cycle may vary and is usually about 1 to 120 seconds, preferably, about 5 to 60 seconds for the denaturation steps, and usually about 1 to 15 seconds, preferably, about 1 to 5 seconds, for the extension steps. It is to be

understood that the actual temperature and duration of the cycles employed are dependent on the particular amplification conducted and are well within the knowledge of those skilled in the art.

Generally, an aqueous medium is employed. Other polar cosolvents may also be  
5 employed, usually oxygenated organic solvents of from 1-6, more usually from 1-4, carbon atoms, including alcohols, ethers, formamide and the like. Usually, these cosolvents, if used, are present in less than about 70 weight percent, more usually in less than about 30 weight percent.

The pH for the medium is usually in the range of about 4.5 to 9.5, more usually in the  
10 range of about 5.5 to 8.5, and preferably in the range of about 6 to 8. Various buffers may be used to achieve the desired pH and maintain the pH during the determination. Illustrative buffers include borate, phosphate, carbonate, Tris, barbital and the like. The particular buffer employed is not critical to this invention but in individual methods one buffer may be preferred over another. The medium may also contain materials required for enzyme activity  
15 such as a divalent metal ion (usually magnesium).

The selection of the snp detection sequence will affect the stringency employed during the primer extension, particularly at the stage of hybridization. Since in a substantial number of samples, the DNA will be heterozygous for snps, rather than homozygous, one does not wish to have false positives, where the snp detection sequence may bond to the sequence  
20 comprising the prevalent nucleotide, as well as the sequence comprising the snp. Where the DNA sample is homozygous for the prevalent sequence, it is also important that the snp detection sequence does not bind to give a false positive. Therefore, the difference in  $T_m$  between the snp containing sequence and the wild-type sequence will usually be at least about 3°C, more usually at least about 5°C, under the conditions of the primer extension.

25 Various ancillary materials will frequently be employed in the methods in accordance with the present invention. For example, in addition to buffers and salts, the medium may also comprise stabilizers for the medium and the reaction components. Frequently, the medium may also include proteins such as albumins, quaternary ammonium salts, polycations such as spermine, surfactants, particularly non-ionic surfactants, binding enhancers, e.g.,  
30 polyalkylene glycols, or the like.

The reaction is conducted for a time sufficient to produce the desired number of copies of each of the polynucleotides suspected of being present as discussed below. Generally, the time period for conducting the entire method will be from about 10 to 200 minutes. As mentioned above, it is usually desirable to minimize the time period.

5       The concentration of the nucleotide polymerase is usually determined empirically. Preferably, a concentration is used that is sufficient such that the amplification is robust. The primary limiting factor generally is the cost of the reagent. Such enzymes include Pfu DNA polymerase (native and recombinant) from Stratagene, La Jolla, CA, Ultma DNA polymerase from Perkin Elmer, Foster City, CA, rBst DNA polymerase from Epicentre Technologies, 10 Madison, WI, VENT DNA polymerase from New England Biolabs, Beverly, MA, Tli DNA polymerase from Promega Corp., Madison, WI, and Pwo DNA polymerase from Boehringer Mannheim, Indianapolis; IN, and the like.

15      The initial concentration of each of the polynucleotides containing the respective target snps can be as low as about 50 picograms per microliter in a sample. After amplification the concentration of each polynucleotide should be at least about 10 picomolar, generally in the range of about 10 picomolar to about 10 nanomolar, usually from about 10 to  $10^{10}$ , more usually from about  $10^3$  to  $10^8$  molecules in a sample preferably at least  $10^{-21}$ M in the sample and may be  $10^{-10}$  to  $10^{-19}$ M, more usually  $10^{-14}$  to  $10^{-19}$ M. In general, the reagents for the reaction are provided in amounts to achieve extension of the oligonucleotide primers.

20      The concentration of the oligonucleotide primer(s) will be about 1 to about 20 micromolar and is usually about 1 to about 10 micromolar, preferably, about 1 to about 4 micromolar, for a sample size that is about 10 femtomolar. Preferably, the concentration of the oligonucleotide primer(s) is substantially in excess over, preferably at least about  $10^7$  to about  $10^{10}$  times greater than, more preferably, at least about  $10^9$  times greater than, the concentration of the corresponding target polynucleotides.

25      The amount of the oligonucleotide probes will be 10 to about 500 nanomolar and is usually about 50 to about 200 nanomolar for a sample size that is about 10 femtomolar (10 femtograms per microliter). Preferably, the concentration of the oligonucleotide probes is substantially in excess over, preferably at least about  $10^7$  times greater than, more preferably, at least about  $10^8$  times greater than, the concentration of each of the target polynucleotides.

The concentration of the nucleoside triphosphates in the medium can vary widely; preferably, these reagents are present in an excess amount. The nucleoside triphosphates are usually present in about 100 micromolar to about 1 millimolar, preferably, about 20 to about 400 micromolar.

5       The order of combining of the various reagents to form the combination may vary. Usually, the sample containing the polynucleotides is combined with a pre-prepared combination of nucleoside triphosphates and nucleotide polymerase. The oligonucleotide primers and the snp detection sequences may be included in the prepared combination or may be added subsequently. However, simultaneous addition of all of the above, as well as other  
10 step-wise or sequential orders of addition, may be employed provided that all of the reagents described above are combined prior to the start of the reactions. The oligonucleotide pairs may be added to the combination of the reagents at or prior to the initiation of the primer extension reaction and may be replenished from time-to-time during the primer extension reaction.

15       For quantitation, one may choose to use controls, which provide a signal in relation to the amount of the target that is present or is introduced. Where one is dealing with a mixture of nucleic acid molecules, as in the case of mRNA in a lysate, one may use the known amounts of one or more different mRNAs in the particular cell types as the standards. Desirably, one would have at least two controls, preferably at least 3 controls, where the  
20 variation in number between any two controls is at least about  $10^2$ , and the total range is at least about  $10^3$ , usually at least about  $10^4$ . However, determining the consistent ratio of mRNAs occurring naturally will result in a large margin of error, so that one would usually rely on synthetic targets. Where a control system is added for quantitation, as compared to relying on the presence of a known amount of a plurality of endogenous nucleic acids, the  
25 control system will comprise at least two control sequences, usually at least 3 control sequences and generally not more than about 6 control sequences, where the upper limit is primarily one of convenience and economy, since additional control sequences will usually not add significant additional precision, which will usually be at least about 50nucleotides, more usually at least about 100nucleotides. The control sequences will have a common primer  
30 sequence and different control detection sequences, which are intended to parallel the primer

sequence and snp detection sequence in size, spacing and response to the primer extension conditions. In carrying out the primer extension reaction with sample nucleic acid, one would then add different number of molecules of the different control sequences, so that one could graph the result to give a signal/number relationship. This graph could then be used to relate signals observed with target molecules to the number of molecules present.

After completion of the primer extension reaction, either by monitoring the change in fluorescence as described above or taking aliquots and assaying for total free electrophoretic tags, the mixture may now be analyzed. Depending on the instrument, today from one to four different fluorescers activated by the same light source and emitting at different detectable labels may be used. With improvements, five or more different fluorescers will be available, where an additional light source may be required. Electrochemical detection is described in U.S. Patent no. 6,045,676.

In those instances where a fluorescent label is not present on the electrophoretic tag bound to the snp detection sequence, the mixture may be added to functionalized fluorescent tags to label the electrophoretic tag with a fluorescer. For example, where a thiol group is present, the fluorescer could have an activated ethylene, such as maleic acid to form the thioether. For hydroxyl groups, one could use activated halogen or pseudohalogen for forming an ether, such as an  $\alpha$ -haloketone. For carboxyl groups, carbodiimide and appropriate amines or alcohols would form amides and esters, respectively. For an amine, one could use activated carboxylic acids, aldehydes under reducing conditions, activated halogen or pseudohalogen, etc. When synthesizing oligopeptides, protective groups are used. These could be retained while the fluorescent moiety is attached to an available functionality on the oligopeptide.

The presence of each of the cleaved electrophoretic tags is determined by the label. The separation of the mixture of labeled electrophoretic tags is carried out by electroseparation, which involves the separation of components in a liquid by application of an electric field, preferably, by electrokinetics (electrokinetic flow), electrophoretic flow, electroosmotic flow or combination thereof, with the separation of the E-TAG mixture into individual fractions or bands. Electroseparation involves the migration and separation of molecules in an electric field based on differences in mobility. Various forms of

electroseparation include, by way of example and not limitation, free zone electrophoresis, gel electrophoresis, isoelectric focusing and isotachophoresis. Capillary electroseparation involves electroseparation, preferably by electrokinetic flow, including electrophoretic, dielectrophoretic and/or electroosmotic flow, conducted in a tube or channel of about 1-200 micrometer, usually, about 10-100 micrometers cross-sectional dimensions. The capillary may be a long independent capillary tube or a channel in a wafer or film comprised of silicon, quartz, glass or plastic.

In capillary electroseparation, an aliquot of the reaction mixture containing the cleaved labels is subjected to electroseparation by introducing the aliquot into an electroseparation channel that may be part of, or linked to, a capillary device in which the amplification and other reactions are performed. An electric potential is then applied to the electrically conductive medium contained within the channel to effectuate migration of the components within the combination. Generally, the electric potential applied is sufficient to achieve electroseparation of the desired components according to practices well known in the art. One skilled in the art will be capable of determining the suitable electric potentials for a given set of reagents used in the present invention and/or the nature of the cleaved labels, the nature of the reaction medium and so forth. The parameters for the electroseparation including those for the medium and the electric potential are usually optimized to achieve maximum separation of the desired components. This may be achieved empirically and is well within the purview of the skilled artisan.

Capillary devices are known for carrying out amplification reactions such as PCR. See, for example, Analytical Chemistry (1996) 68:4081-4086. Devices are also known that provide functional integration of PCR amplification and capillary electrophoresis in a microfabricated DNA analysis device. One such device is described by Woolley, *et al.*, in Anal. Chem. (1996) 68:4081-4086. The device provides a microfabricated silicon PCR reactor and glass capillary electrophoresis chips. In the device a PCR chamber and a capillary electrophoresis chip are directly linked through a photolithographically fabricated channel filled with a sieving matrix such as hydroxyethylcellulose. Electrophoretic injection directly from the PCR chamber through the cross injection channel is used as an "electrophoretic valve" to couple the PCR and capillary electrophoresis devices on a chip.

The capillary electrophoresis chip contains a sufficient number of main or secondary electrophoretic channels to receive the desired number of aliquots from the PCR reaction medium or the solutions containing the cleaved labels, etc., at the intervals chosen.

For capillary electrophoresis one may employ one or more detection zones to detect the separated cleaved labels. It is, of course, within the purview of the present invention to utilize several detection zones depending on the nature of the amplification process, the number of cycles for which a measurement is to be made and so forth. There may be any number of detection zones associated with a single channel or with multiple channels. Suitable detectors for use in the detection zones include, by way of example, photomultiplier tubes, photodiodes, photodiode arrays, avalanche photodiodes, linear and array charge coupled device (CCD) chips, CCD camera modules, spectrofluorometers, and the like. Excitation sources include, for example, filtered lamps, LED's, laser diodes, gas, liquid and solid state lasers, and so forth. The detection may be laser scanned excitation, CCD camera detection, coaxial fiber optics, confocal back or forward fluorescence detection in single or array configurations, and the like.

Detection may be by any of the known methods associated with the analysis of capillary electrophoresis columns including the methods shown in U.S. Patent Nos. 5,560,811 (column 11, lines 19-30), 4,675,300, 4,274,240 and 5,324,401, the relevant disclosures of which are incorporated herein by reference.

Those skilled in the electrophoresis art will recognize a wide range of electric potentials or field strengths may be used, for example, fields of 10 to 1000 V/cm are used with 200-600 V/cm being more typical. The upper voltage limit for commercial systems is 30 kV, with a capillary length of 40-60 cm, giving a maximum field of about 600 V/cm. For DNA, typically the capillary is coated to reduce electroosmotic flow, and the injection end of the capillary is maintained at a negative potential.

For ease of detection, the entire apparatus may be fabricated from a plastic material that is optically transparent, which generally allows light of wavelengths ranging from 180 to 1500 nm, usually 220 to 800 nm, more usually 450 to 700nm, to have low transmission losses. Suitable materials include fused silica, plastics, quartz, glass, and so forth.

In mass spectrometry, the E-TAGs may be different from the E-TAGs used in electrophoresis, since the E-TAGs do not require a label, nor a charge. Thus, these E-TAGs may be differentiated solely by mass, which can be a result of atoms of different elements, isotopes of such elements, and numbers of such atoms. In the subject invention, such use of

5 E-TAGs will be coupled with a process for removing the iterative extensions of the nucleic acid sequence, where degradation or cleavage has occurred at a site other than the ultimate linkage.

The electrophoretic tag for use in electrophoresis may be represented by the formula:



10 wherein R is a label, particularly a fluorescer, L is a bond or a linking group as described previously, and T comprises a nucleoside base, purine or pyrimidine, and is the base, a nucleoside, nucleotide or nucleotide triphosphate, when the base is retained and is otherwise a functionality resulting from the cleavage. L provides a major factor in the differences in mobility between the different electrophoretic tags, in combination with the label and any

15 nucleotides, which remain with the linker. L may or may not include a cleavable linker, depending upon whether the terminal nucleotide(s) are to be retained or completely removed.

The linker L may include charged groups, uncharged polar groups or be non-polar. The groups may be alkylene and substituted alkynes, oxyalkylene and polyoxyalkylene, particularly alkylene of from 2 to 3 carbon atoms, arylanes and substituted arylanes,

20 polyamides, polyethers, polyalkylene amines, etc. Substituents may include heteroatoms, such as halo, phosphorous, nitrogen, oxygen, sulfur, etc., where the substituent may be halo, nitro, cyano, non-oxo-carbonyl, e.g. ester, acid and amide, oxo-carbonyl, e.g. aldehyde and keto, amidine, urea, urethane, guanidine, carbamyl, amino and substituted amino, particularly alkyl substituted amino, azo, oxy, e.g. hydroxyl and ether, etc., where the substituents will

25 generally be of from about 0 to 10 carbon atoms, while the linking group will generally be of from about 1 to 100 carbon atoms, more usually of from about 1 to 60 carbon atoms and preferably about 1 to 36 carbon atoms. The linking group will be joined to the label and the nucleotide by any convenient functionality, such as carboxy, amino, oxy, phospo, thio, iminoether, etc., where in many cases the label and the nucleotide will have a convenient

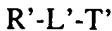
30 functionality for linkage.

The number of heteroatoms in L is sufficient to impart the desired charge to the label conjugate, usually from about 1 to about 200, more usually from about 2 to 100, heteroatoms. The heteroatoms in L may be substituted with atoms other than hydrogen.

The charge-imparting moieties of L may be, for example, amino acids,

5 tetraalkylammonium, phosphonium, phosphate diesters, carboxylic acids, thioacids, sulfonic acids, sulfate groups, phosphate monoesters, and the like and combinations of one or more of the above. The number of the above components of L is such as to achieve the desired number of different charge-imparting moieties. The amino acids may be, for example, lysine, aspartic acid, alanine, gamma-aminobutyric acid, glycine,  $\beta$ -alanine, cysteine, glutamic acid, 10 homocysteine,  $\beta$ -alanine and the like. The phosphate diesters include, for example, dimethyl phosphate diester, ethylene glycol linked phosphate diester, and so forth. The thioacids include, by way of example, thioacetic acid, thiopropionic acid, thiobutyric acid and so forth. The carboxylic acids preferably have from 1 to 30 carbon atoms, more preferably, from 2 to 15 carbon atoms and preferably comprise one or more heteroatoms and may be, for example, 15 acetic acid derivatives, formic acid derivatives, succinic acid derivatives, citric acid derivatives, phytic acid derivatives and the like. In one embodiment of the present invention the label conjugates having different charge to mass ratios may comprise fluorescent compounds, each of which are linked to molecules that impart a charge to the fluorescent compound conjugate. As indicated previously, desirably the linking group has an overall negative charge, preferably having in the case of a plurality of groups, groups of the same charge, where the total charge may be reduced by having one or more positively charged 20 moiety.

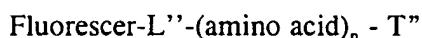
Combinations of particular interest comprise a fluorescent compound and a different amino acid or combinations thereof in the form of a peptide or combinations of amino acids 25 and thioacids or other carboxylic acids. Such compounds are represented by the formula:



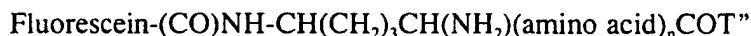
wherein R' is a fluorescer, L' is an amino acid or a peptide or combinations of amino acids and thioacids or other carboxylic acids and T' is a functionality for linking to a nucleoside base or is a nucleoside, nucleotide or nucleotide triphosphate.

In a preferred embodiment of the present invention, the charge-imparting moiety is conveniently composed primarily of amino acids but also may include thioacids and other carboxylic acids having from one to five carbon atoms. The charge imparting moiety may have from 1 to 30, preferably 1 to 20, more preferably, 1 to 10 amino acids per moiety and may also 5 comprise 1 to 3 thioacids or other carboxylic acids. As mentioned above, any amino acid, both naturally occurring and synthetic may be employed.

In a particular embodiment the label conjugates may be represented by the formula:



wherein L'' is a bond or a linking group of from 1 to 20 atoms other than hydrogen, n is 1 to 10, and T'' comprises a nucleoside base, purine or pyrimidine, including a base, a nucleoside, a nucleotide or nucleotide triphosphate. An example of label conjugates in this embodiment, by way of illustration and not limitation, is one in which the fluorescer is fluorescein, L'' is a bond in the form of an amide linkage involving the meta-carboxyl of the fluorescein and the terminal amine group of lysine, and T'' is a nucleotide triphosphate. These label conjugates 15 may be represented as follows:



wherein X is as set forth in Table 1.

Table 1

	No.	X	Charge
20	1	OH	-2
	2	NH-lysine	-1
25	3	NH-(lysine) <sub>2</sub>	neutral
	4	NH-alanine	-3
	5	NH-aspartic acid	-4
	6	NH-(aspartic acid) <sub>2</sub>	-5
	7	NH-(aspartic acid) <sub>3</sub>	-6
	8	NH-(aspartic acid) <sub>4</sub>	-7
30	9	NH-(aspartic acid) <sub>5</sub>	-8
	10	NH-(aspartic acid) <sub>6</sub>	-9
	11	NH-(aspartic acid) <sub>7</sub>	-10
	12	NH-alanine-lysine	-2 (unique q/M)
	13	NH-aspartic acid-lysine	-3 (unique q/M)
35	14	NH-(aspartic acid) <sub>2</sub> -lysine	-4 (unique q/M)
	15	NH-(aspartic acid) <sub>3</sub> -lysine	-5 (unique q/M)

16	NH-(aspartic acid) <sub>4</sub> -lysine	-6 (unique q/M)
17	NH-(aspartic acid) <sub>5</sub> -lysine	-7 (unique q/M)
18	NH-(aspartic acid) <sub>6</sub> -lysine	-8 (unique q/M)
19	NH-(aspartic acid) <sub>7</sub> -lysine	-9 (unique q/M)
5 20	NH-(aspartic acid) <sub>8</sub> -lysine	-10 (unique q/M)
21	NH-(lysine) <sub>4</sub>	1
22	NH-(lysine) <sub>5</sub>	+2

wherein q is charge, M is mass and mobility is  $q/M^{2/3}$ . Examples of such label conjugates are  
10 shown in Fig. 1C.

Table 2 shows various characteristics for the label conjugates.

15 Table 2

No.	Mass(M)	Charge(q)	$M^{2/3}$	$q/M^{2/3}$	Mobility
1	744.82	0	82.16765	0	0
2	877.02	0	91.62336	0	0
3	828.71	-1	88.22704	-0.01133	-0.16546
20 4	970.71	-1	98.03767	-0.0102	-0.1489
5	700.82	-2	78.89891	-0.02535	-0.37004
6	842.83	-2	89.22639	-0.2241	-0.32721
7	815.92	-3	87.31692	-0.03436	-0.50155
25 8	957.92	-3	97.17461	-0.03087	-0.45067
9	931.02	-4	95.34677	-0.04195	-0.61242
10	1073.02	-4	104.8106	-0.03816	-0.55712
11	1046	-5	103.0436	-0.04852	-0.70834
12	1188	-5	112.1702	-0.04458	-0.65071
13	1161	-6	110.4642	-0.05432	-0.79291
30 14	1303	-6	119.297	-0.05029	-0.7342
15	1276	-7	117.6433	-0.0595	-0.86861
16	1418	-7	126.2169	-0.05546	-0.80961
17	1391	-8	124.6096	-0.0642	-0.9372
35 18	1533	-8	132.952	-0.06017	-0.87839
19	1506	-9	131.3863	-0.0685	-0.99997
20	1648	-9	139.6205	-0.06451	-0.94167
21	793.52	1	85.7114	0.011667	0.170316
22	935.52	1	95.65376	0.010454	0.152613

40 The label conjugates may be prepared utilizing conjugating techniques that are well known in the art. The charge-imparting moiety L may be synthesized from smaller molecules

that have functional groups that provide for linking of the molecules to one another, usually in a linear chain. Such functional groups include carboxylic acids, amines, and hydroxy- or thiol-groups. In accordance with the present invention the charge-imparting moiety may have one or more side groups pending from the core chain. The side groups have a functionality to 5 provide for linking to a label or to another molecule of the charge-imparting moiety.

Common functionalities resulting from the reaction of the functional groups employed are exemplified by forming a covalent bond between the molecules to be conjugated. Such 10 functionalities are disulfide, amide, thioamide, dithiol, ether, urea, thiourea, guanidine, azo, thioether, carboxylate and esters and amides containing sulfur and phosphorus such as, e.g. sulfonate, phosphate esters, sulfonamides, thioesters, etc., and the like.

The chemistry for performing the types of syntheses to form the charge-imparting 15 moiety as a peptide chain is well known in the art. See, for example, Marglin, *et al.*, Ann. Rev. Biochem. (1970) 39:841-866. In general, such syntheses involve blocking, with an appropriate protecting group, those functional groups that are not to be involved in the 20 reaction. The free functional groups are then reacted to form the desired linkages. The peptide can be produced on a resin as in the Merrifield synthesis (Merrifield, J. Am. Chem. Soc. (1980) 85:2149-2154 and Houghten *et al.*, Int. J. Pep. Prot. Res. (1980) 16:311-320. The peptide is then removed from the resin according to known techniques.

A summary of the many techniques available for the synthesis of peptides may be 25 found in J. M. Stewart, *et al.*, "Solid Phase Peptide Synthesis", W. H. Freeman Co, San Francisco (1969); and J. Meienhofer, "Hormonal Proteins and Peptides", (1973), vol. 2, p. 46., Academic Press (New York), for solid phase peptide synthesis and E. Schroder, *et al.*, "The Peptides, vol. 1, Academic Press (New York), 1965 for solution synthesis.

In general, these methods comprise the sequential addition of one or more amino acids, 30 or suitably protected amino acids, to a growing peptide chain. Normally, a suitable protecting group protects either the amino or carboxyl group of the first amino acid. The protected or derivatized amino acid can then be either attached to an inert solid support or utilized in solution by adding the next amino acid in the sequence having the complementary (amino or carboxyl) group suitably protected, under conditions suitable for forming the amide linkage. The protecting group is then removed from this newly added amino acid residue and the next

amino acid (suitably protected) is then added, and so forth. After all the desired amino acids have been linked in the proper sequence, any remaining protecting groups (and any solid support) are removed sequentially or concurrently, to afford the final peptide. The protecting groups are removed, as desired, according to known methods depending on the particular 5 protecting group utilized. For example, the protecting group may be removed by reduction with hydrogen and palladium on charcoal, sodium in liquid ammonia, etc.; hydrolysis with trifluoroacetic acid, hydrofluoric acid, and the like.

After the synthesis of the peptide is complete, the peptide is removed from the resin by conventional means such as ammonolysis, acidolysis and the like. The fully deprotected 10 peptide may then be purified by techniques known in the art such as chromatography, for example, adsorption chromatography; ion exchange chromatography, partition chromatography, high performance liquid chromatography, thin layer chromatography, and so forth.

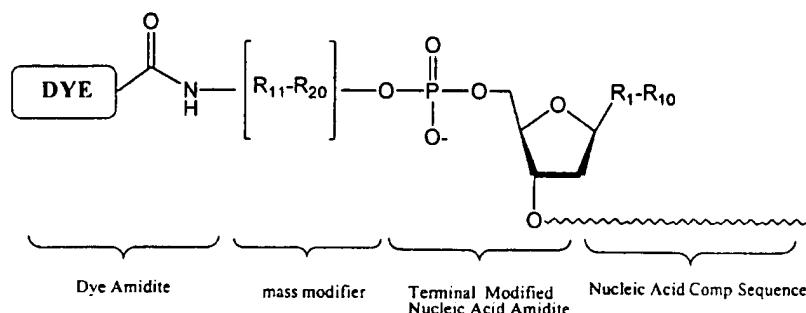
As can be seen, the selected peptide representing a charge-imparting moiety may be 15 synthesized separately and then attached to the label either directly or by means of a linking group. On the other hand, the peptide may be synthesized as a growing chain on the label. In any of the above approaches, the linking of the peptide or amino acid to the label may be carried out using one or more of the techniques described above for the synthesis of peptides or for linking moieties to labels.

The aforementioned label conjugates with different electrophoretic mobility permit a multiplexed amplification and detection of multiple single nucleotide polymorphisms. The label conjugates are linked to oligonucleotides in a manner similar to that for labels in general, by means of linkages that are enzymatically cleavable. It is, of course, within the purview of the present invention to prepare any number of label conjugates for performing multiplexed 20 determinations. Accordingly, for example, with 40 to 50 different label conjugates separated in a single separation channel and 96 different amplification reactions with 96 separation channels on a single plastic chip, one can detect 4000 to 5000 single nucleotide polymorphisms.

As exemplary of the subject invention, four target polynucleotides T1, T2, T3 and T4 30 are employed. Oligonucleotide primers PR1, PR2, PR3 and PR4 are employed, each

respectively capable of hybridizing to a sequence in the respective target polynucleotides. Also employed are four oligonucleotide snp detection sequences, PB1, PB2, PB3 and PB4. Each of the snp detection sequences comprises a fluorescent label F1, F2, F3 and F4, respectively. In this example, there is a mismatch between PB2 and T2, which comprises a 5 single nucleotide polymorphism. The reaction medium comprising the above reagents and nucleoside triphosphates and a template dependent polynucleotide polymerase having 5' to 3' exonuclease activity is treated under amplification conditions. Primers PR1, PR2, PR3 and PR4 hybridize to their respective target polynucleotides and are extended to yield extended primers EPR1, EPR2, EPR3 and EPR4. snp detection sequences PB1, PB3 and PB4, which 10 hybridize with their respective target polynucleotides, are acted upon by the exonuclease to cleave a single nucleotide bearing the respective fluorescent label. PB2, which does not bind to the target polynucleotide, is not cleaved. Cleaved fragments F1, F3 and F4 are injected into a separation channel in a chip for conducting electroseparation. The labels are identified by their specific mobility and fluorescence upon irradiation. The separated labels are related 15 to the presence and amount of the respective target polynucleotide.

Synthesis of electrophoretic tags can be most easily and effectively achieved via assembly on a solid phase support during probe synthesis using standard phosphoramidite chemistries. The concept hinges on the formation of a unique electrophoretic tags during an S<sub>1</sub> nuclease digest of the probe. It is important to note that only the 5'-terminal base paired 20 residue of the probe is cleaved. As such, any components bound to the terminal residue which do not form base pairs will be released as well. Therefore, electrophoretic tags can be assembled by the coupling of a penultimate unique base pairing residue followed by a final coupling of a dye residue containing a phosphoramidite handle. The electrophoretic tags is thus composed of two units of variable charge to mass ratios bridged by a phosphate linker 25 (See below).



The design and synthesis of electrophoretic tags on solid phase support using standard phosphoramidite coupling chemistry.

5       The separation of electrophoretic tags, which differ by 9 mass units (Table 1) has been demonstrated **Figure 7**. The penultimate coupling during probe synthesis is initially carried out using commercially available modified (and unmodified) phosphoramidites (Table 2). This residue is able to form hydrogen bonds to its partner in the target strand and is considered a mass modifier but could potentially be a charge modifier as well. The phosphate 10 bridge formed during this coupling is the linkage severed during the 5'-nuclease assay. The final coupling is done using a phosphoramidite analogue of a dye. Fluorescein is conveniently employed, but other dyes can be used as well.

15      The initial synthetic approach is outlined in **Scheme 1**. Starting with commercially available 6-carboxy fluorescein, the phenolic hydroxyl groups are protected using an anhydride. Isobutyric anhydride in pyridine was employed but other variants are equally suitable. It is important to note the significance of choosing an ester functionality as the protecting group. This species remains intact through the phosphoramidite monomer synthesis as well as during oligonucleotide construction. These groups are not removed until the synthesized oligo is deprotected using ammonia. After protection the crude material is then 20 activated *in situ* via formation of an N-hydroxy succinimide ester (NHS-ester) using DCC as a coupling agent. The DCU byproduct is filtered away and an amino alcohol is added. Many amino alcohols are

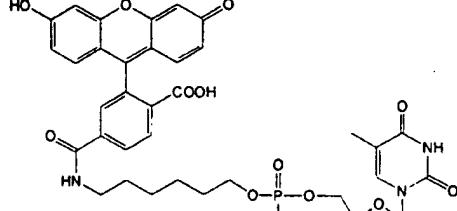
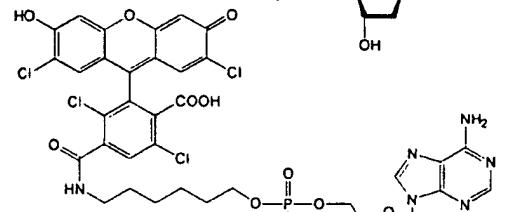
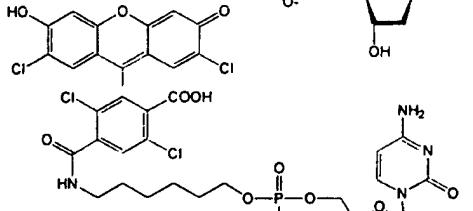
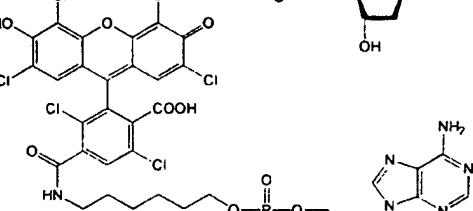
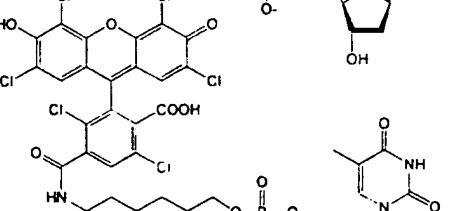
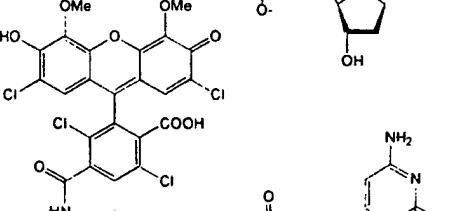
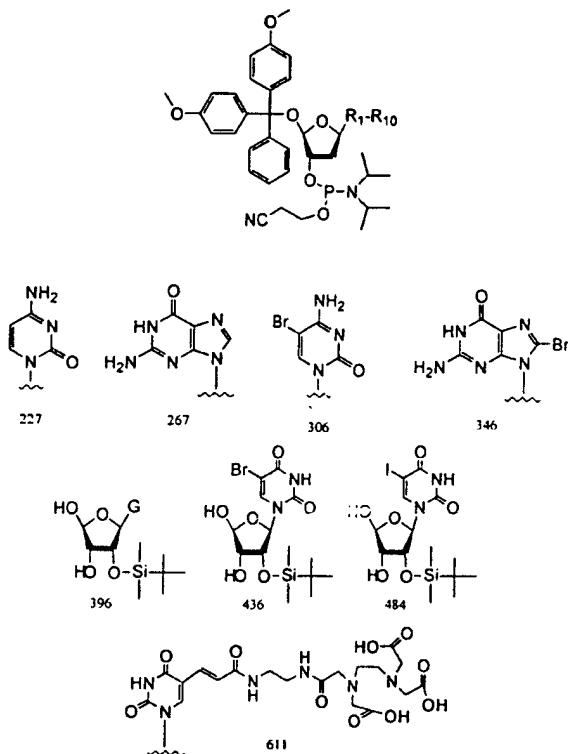
<u>E-Tag</u>	<u>Elution Time on CE (sec)</u>	<u>Mass</u>
	385	778
	428	925
	438	901
	462	994
	480	985
	555	961

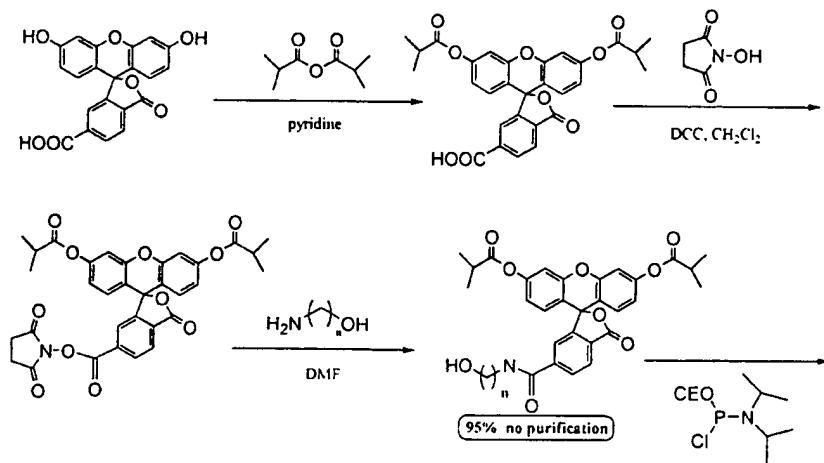
Table 1. E-Tags that have been separated on a LabCard (detection:4.7 cm; 200 V/cm).



**Table 2.** Potential mass and charged modified nucleic acid phosphorimidites to be employed as the penultimate coupling during probe synthesis on a standard DNA synthesizer.

5

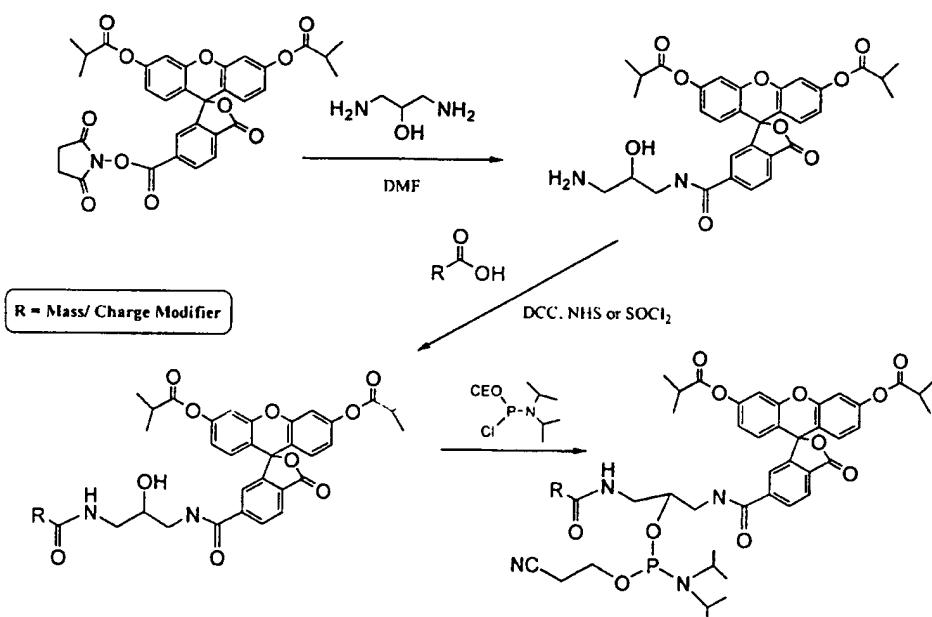
commercially available some of which are derived from reduction of amino acids. Only the amine is reactive enough to displace N-hydroxy succinimide. Upon standard



**Scheme 1.**

extractive workup, a 95% yield of product is obtained. This material is phosphitylated to generate the phosphoramidite monomer (**Scheme 1**). For the synthesis of additional E-Tags, a symmetrical bisamino alcohol linker is used as the amino alcohol (**Scheme 2**). As such the second amine is then coupled with a multitude of carboxylic acid derivatives (**Table 3**) prior to the phosphitylation reaction. Using this methodology hundreds if not thousands of E-TAGs with varying charge to mass ratios can easily be assembled during probe synthesis on a DNA synthesizer using standard chemistries.

10

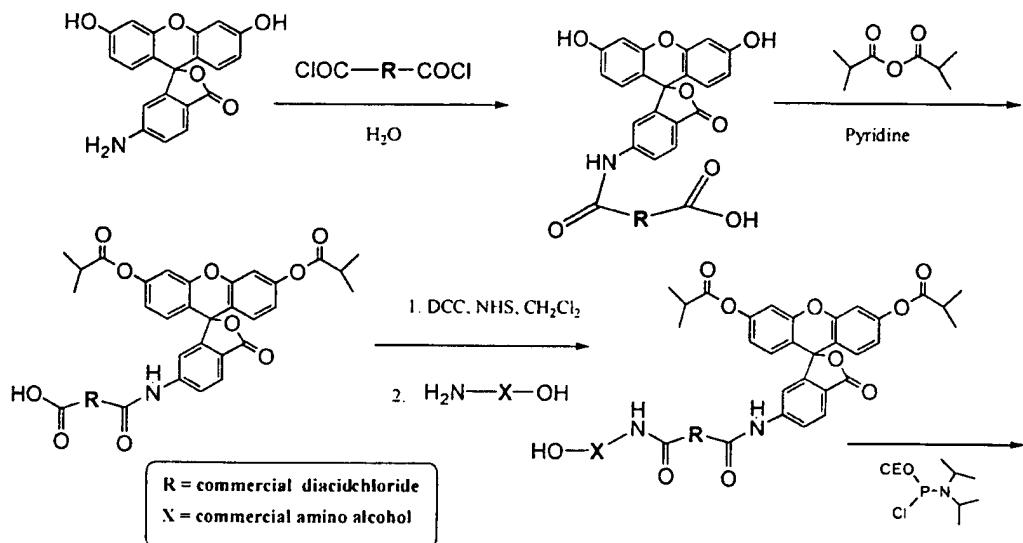
**Scheme 2.**

15

Additional electrophoretic tags are accessed via an alternative strategy which uses 5-aminofluorescein as starting material (**Scheme 3**). Addition of 5-aminofluorescein to a great excess of a diacid chloride in a large volume of solvent allows for the predominant formation of the monoacylated product over dimer formation. The phenolic groups are not reactive under these conditions. Aqueous workup converts the terminal acid chloride to a carboxylic

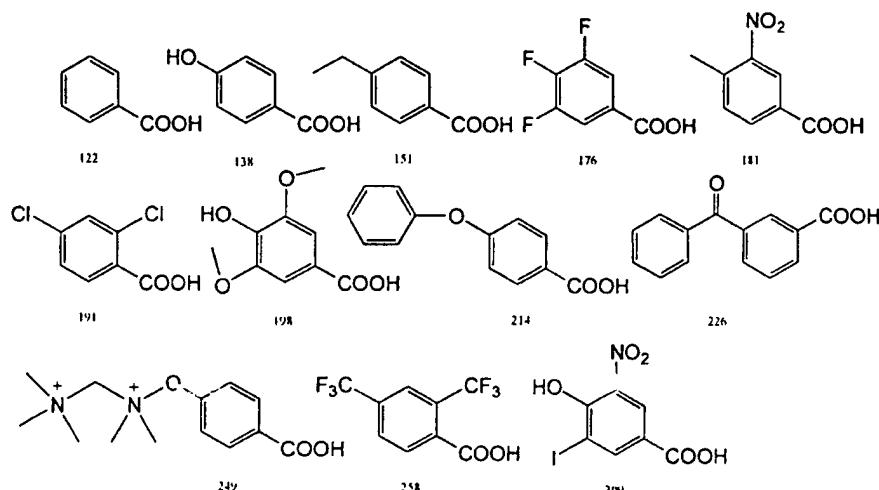
acid. This product is analogous to 6-carboxy fluorescein and using the same series of steps is converted to its protected phosphoramidite monomer (Scheme 3). There are many commercially available di(acid chorides) and diacids, which can be converted to diacid chlorides using  $\text{SOCl}_2$  or acetyl chloride. This methodology is highly attractive in that a second mass modifier is used. As such, if one has access to 10 commercial modified phosphoramidites and 10 diacid chlorides and 10 amino alcohols there is a potential for 1000 different E-Tags. There are many commercial diacid chlorides and amino alcohols (Table 4). These synthetic approaches are ideally suited for combinatorial chemistry.

10



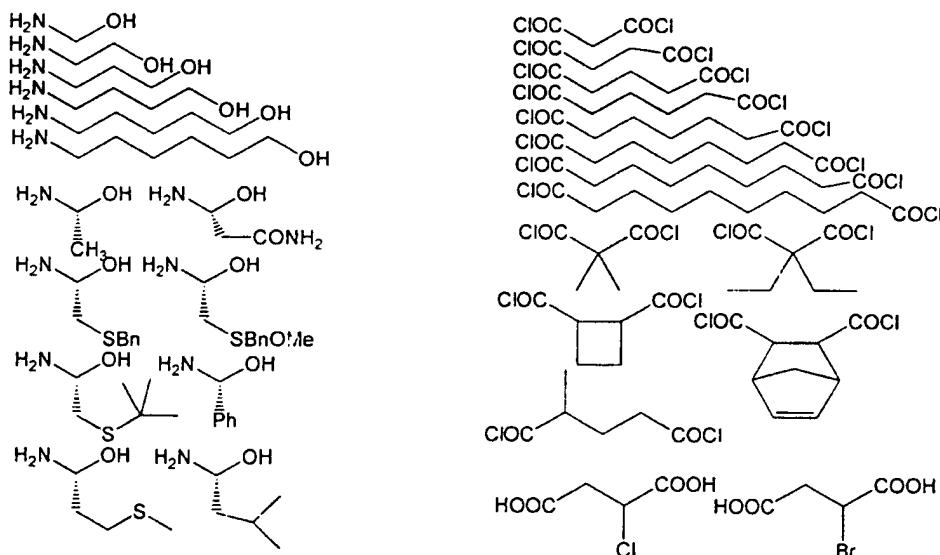
Scheme 3.

15



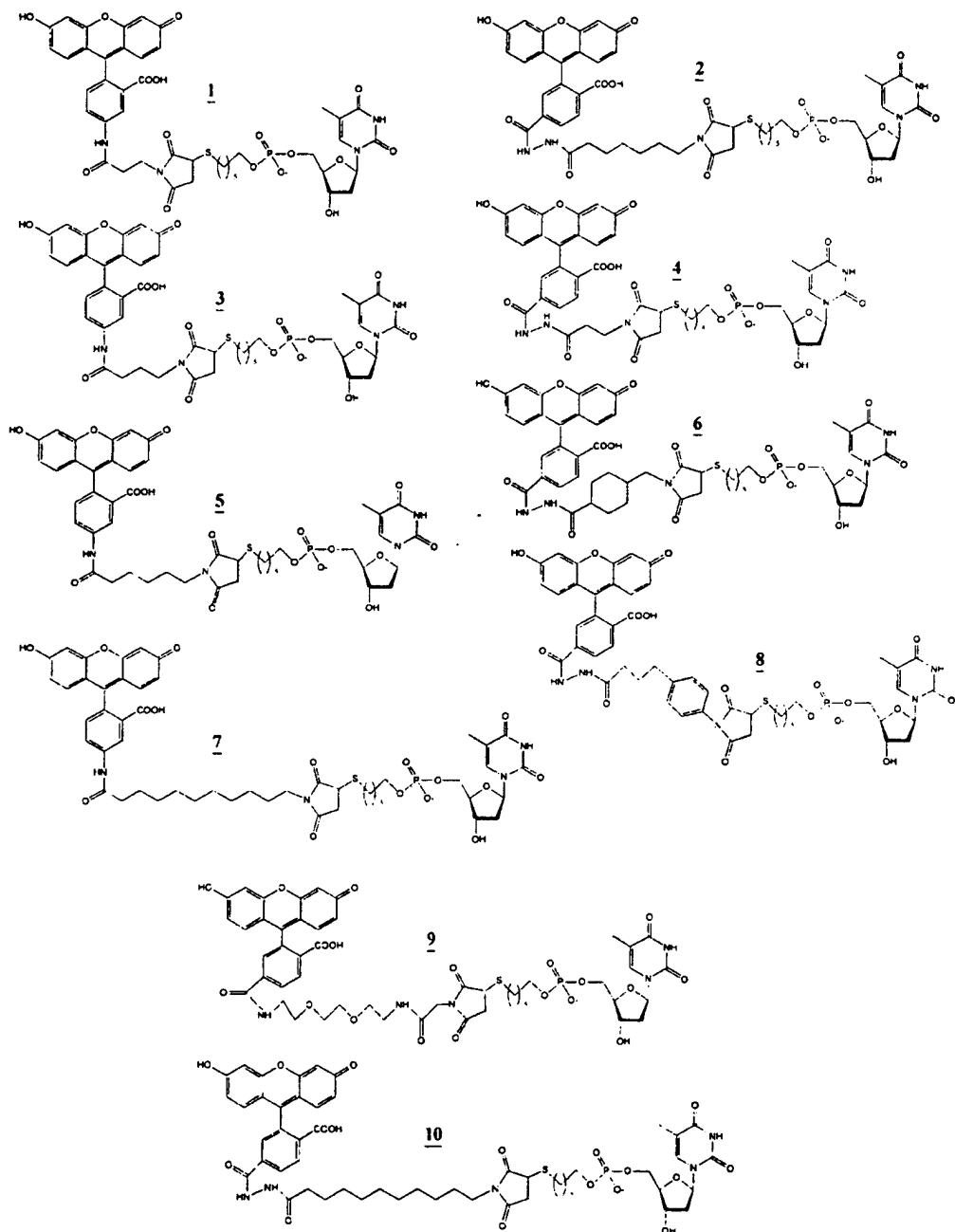
**Table 3.** Benzoic acid derivatives as mass and charge modifiers. (Mass is written below each)

5 modifier)



**Table 4.** Mass and charge modifiers that can be used for conversion of amino dyes into E-Tag phosphoramidite monomers.

A variety of maleimide derivatized E-Tags have also been synthesized. These compounds were subsequently bioconjugated to 5'-thiol adorned DNA sequences and subjected to the 5'-nuclease assay. The species formed upon cleavage are depicted in Table 5.



**Table 5.** Electrophoretic Tags derived from maleimide linked precursors.

As a matter of convenience, predetermined amounts of reagents employed in the present invention can be provided in a kit in packaged combination. A kit can comprise in  
5 packaged combination an oligonucleotide primer for each polynucleotide suspected of being in said set wherein each of said primers is hybridizable to a first sequence of a respective polynucleotide if present, a template dependent polynucleotide polymerase, nucleoside triphosphates, and a set of oligonucleotide snp detection sequences, each of said oligonucleotide probes having a fluorescent label at its 5'-end and having a sequence at its 5'-  
10 end that is hybridizable to a respective polynucleotide wherein each of said labels is cleavable from said oligonucleotide probe.

The kit may further comprise a device for conducting capillary electrophoresis. The label is cleavable by a template dependent polynucleotide polymerase having 5' to 3' exonuclease activity. The kit can further include various buffered media, some of which may  
15 contain one or more of the above reagents.

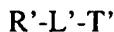
The relative amounts of the various reagents in the kits can be varied widely to provide for concentrations of the reagents necessary to achieve the objects of the present invention. Under appropriate circumstances one or more of the reagents in the kit can be provided as a dry powder, usually lyophilized, including excipients, which on dissolution will provide for a  
20 reagent solution having the appropriate concentrations for performing a method or assay in accordance with the present invention. Each reagent can be packaged in separate containers or some reagents can be combined in one container where cross-reactivity and shelf life permit. The kits may also include a written description of a method in accordance with the present invention as described above.

25 In one embodiment of the kit, the electrophoretic tags are fluorescent conjugates represented by the formula:



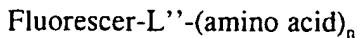
wherein R is a fluorescer, L is a linking group, as described previously, and T is a functionality for binding to a nucleoside base, purine or pyrimidine, or a nucleoside base, a  
30 nucleoside, nucleotide or nucleotide triphosphate.

In another embodiment of a kit, the electrophoretic tags are fluorescent conjugates represented by the formula:



wherein R' is a fluorescer, L' is a bond an amino acid or a peptide or combinations of amino acids and thioacids or other carboxylic acids and T' is a nucleotide or nucleotide triphosphate

In another embodiment of a kit, the electrophoretic tag is a fluorescent conjugate represented by the formula:



wherein L'' is a bond or a linking group of from 1 to 20 atoms in the chain and n is 1 to 100.

The fluorescer may be fluorescein, the amino acid may be lysine and L'' may be a bond in the form of an amide linkage involving the meta-carboxyl of the fluorescein and the terminal amine group of lysine.

In another embodiment of a kit in accordance with the invention , the electrophoretic tag is a label conjugate represented by the formula:



wherein X is selected from the group consisting of: OH, NH-lysine, NH-(lysine)<sub>2</sub>, NH-alanine, NH-aspartic acid, NH-(aspartic acid)<sub>2</sub>, NH-(aspartic acid)<sub>3</sub>, NH-(aspartic acid)<sub>4</sub>, NH-(aspartic acid)<sub>5</sub>, NH-(aspartic acid)<sub>6</sub>, NH-(aspartic acid)<sub>7</sub>, NH-alanine-lysine, NH-aspartic acid-lysine, NH-(aspartic acid)<sub>2</sub>-lysine, NH-(aspartic acid)<sub>3</sub>-lysine, NH-(aspartic acid)<sub>4</sub>-

lysine, NH-(aspartic acid)<sub>5</sub>-lysine, NH-(aspartic acid)<sub>6</sub>-lysine, NH-(aspartic acid)<sub>7</sub>-lysine, NH-(aspartic acid)<sub>8</sub>-lysine, NH-(lysine)<sub>4</sub>, and NH-(lysine)<sub>5</sub>.

The kits will usually have at least about 5 different electrophoretic tags for conjugation, more usually at least about 10, frequently at least about 25 and may have 50 or more, usually not more than about 1,000. The electrophoretic tags will differ as to mobility, including mass/charge ratio and nature of charge, e.g. overall positive or negative, detectable moiety, e.g. fluorophore, electrochemical, etc, or functionality for linking a detectable moiety, e.g. maleimide, mercaptan, aldehyde, ketone, etc.

The electrophoretic tags described above may terminate in an appropriate functionality for linking to a nucleotide or nucleotide triphosphate or may terminate in such moieties.

EXAMPLES

The invention is demonstrated further by the following illustrative examples. Parts and  
5 percentages are by weight unless otherwise indicated. Temperatures are in degrees  
Centigrade (°C) unless otherwise specified. The following preparations and examples  
illustrate the invention but are not intended to limit its scope. Unless otherwise indicated,  
oligonucleotides and peptides used in the following examples were prepared by synthesis using  
an automated synthesizer and were purified by gel electrophoresis or HPLC.

10 The following abbreviations have the meanings set forth below:

Tris HCl - Tris(hydroxymethyl)aminomethane-HCl (a 10X solution) from  
BioWhittaker, Walkersville, MD

HPLC - high performance liquid chromatography

BSA - bovine serum albumin from Sigma Chemical Company, St. Louis MO

15 EDTA - ethylenediaminetetraacetate from Sigma Chemical Company

bp - base pairs

g - grams

mM - millimolar

TET - tetrachlorofluorescein

20 FAM - fluorescein

TAMRA - tetramethyl rhodamine

Reagents:

TET and FAMRA were purchased from Perkin Elmer (Foster City, CA) as were  
25 conjugates of TET, FAM and TAMRA with oligonucleotides.

Master Mix (2X): 20mM Tris-HCl, 2.0mM EDTA, pH 8.0 (8% Glycerol), 10mM MgCl<sub>2</sub>,  
dATP 400μM, dCTP 400μM, dGTP 400μM, dUTP 400μM, AmpliTaq Gold ® 0.1U/μl (from  
Perkin Elmer), Amperase UNG® 0.02U/μl (from Perkin Elmer)

Probes and Primers: (10X)

30 Forward Primer: 3.5μM 5'-TCA CCA CAT CCC AGT G-3' (SEQ ID NO:1)

Reverse Primer 2.0 $\mu$ M 5'-GAG GGA GGTTG GCTG-3' (SEQ ID NO:2)

Plasmid Allele 1 Probe: 2.0 $\mu$ M (200nM per reaction)

5' TET-CCA GCA ACC AAT GAT GCC CGT T-TAMRA-3' (SEQ ID NO:3)

Plasmid Allele 2 Probe: 2.0 $\mu$ M (200nM per reaction)

5' FAM-CCA GCA AGC ACT GAT GCC TGT T-TAMRA-3' (SEQ ID NO:4)

Target DNA:

Plasmid Attle-1: 10 fg/ $\mu$ l = approximately 100<sup>0</sup> copies/ $\mu$ l

Plasmid Allele-2: 10 fg/ $\mu$ l = approximately 1000 copies/ $\mu$ l

10

### Example 1

The experiment was set up to run in the following fashion (6 samples, a triplicate for Allele1 and another triplicate for Allele-2)

22 $\mu$ l of Mastermix

13  $\mu$ l of probes and primers (both the probes are present)

15

4.0  $\mu$ l of Allele-1 or Allele-2

11  $\mu$ l of buffer (10mM Tris-HCl, 1mM EDTA, pH8.0)

The above volumes were added to a PCR tubes and the reaction mixtures were cycled in the following fashion for 40 cycles.

20

### Initial Steps:

The reaction mixtures were kept at 50°C for 2 minutes for optimal AmpErase UNG activity. The 10 minute, 95°C step was required to activate AmpliTaq Gold DNA Polymerase.

Each of the 40 cycles was performed on a Gene Amp ® system 9600 thermal cycler (Perkin Elmer) in the following fashion:

25

	Melt	Anneal/Extend/Cleave
	15 seconds	60 seconds
	95°C	60°C

Results from experiments with Allele-1 are shown in Fig. 2. CE separation of the reaction products of Allele 1 after 0 and 40 cycles. CE instrument was Beckman P/ACE 5000

with LIF detection. BGE: 2.5% LDD30, 7 M urea, 1xTBE. Capillary: 100  $\mu\text{m}$  i.d., 375  $\mu\text{m}$  o.d.,  $L_c = 27 \text{ cm}$ ,  $L_d = 6.9 \text{ cm}$ . Detection:  $\lambda_{\text{ex}} = 488 \text{ nm}$ ,  $\lambda_{\text{em}} = 520 \text{ nm}$ . Injection: 5 s at 2.0 kV. Field strength: 100 V/cm at room temperature. Peaks: P = unreacted primer, P' = primer product.

5 Results from experiments with Allele-2 are shown in Fig. 3. CE separation of the reaction products of Allele 2 after 0 and 40 cycles. Experimental conditions were as given above for Fig. 2 experiment except for BGE composition: 2.0% LDD30, 1xTBE.

### Example2

10 A multiplexed reaction with both Allele 1 and Allele 2 present in equal ratio

The experiment was set up in the following fashion (3 reaction tubes, a triplicate).

22  $\mu\text{l}$  of Mastermix

13  $\mu\text{l}$  of probes and primers (both of the probes were present)

4.0  $\mu\text{l}$  of Allele-1

15 4.0  $\mu\text{l}$  of Allele-2

7  $\mu\text{l}$  of buffer (10mM Tris-HCl, 1mM EDTA, pH8.0)

The above volumes were added to a PCR tubes and the reaction mixtures were cycled in the following fashion for 40 cycles.

20 Initial Steps:

The reaction mixtures are kept at 50°C for 2 minutes for optimal AmpErase UNG activity. The 10 minute, 95°C step is required to activate AmpliTaq Gold DNA Polymerase.

Each of the 40 cycles is performed in the following fashion

Melt	Anneal/ Extend/ Cleave
------	------------------------

25 15 seconds 60 seconds

95°C 60°C

The results are shown in Fig. 4. CE separation of a 1:1 mixture of the 40 cycles products of Alleles 1 and 2. Experimental conditions were as given above for the experiments of Fig. 2.

Example 3

A multiplexed reaction with both Allele 1 and Allele 2: Allele 1 is 10 times more concentrated than Allele 2.

The experiment was set up in the following fashion (3 reaction tubes, a triplicate)

5            22 µl of Mastermix  
              13 µl of probes and primers (both the probes were present)  
              5.0 µl of Allele 1  
              0.5 µl of Allele 2  
              9.5 µl of buffer (10mM Tris-HCl, 11mM EDTA, pH8.0)  
10          The above volumes were added to respective PCR tubes and the reaction mixtures were cycled in the following fashion for 40 cycles.

Initial Steps:

The reaction mixtures were kept at 50°C for 2 minutes for optimal AmpErase UNG activity. The 10 minute 95°C step was required to activate AmpliTaq Gold DNA Polymerase. Each of the 40 cycles is performed in the following fashion

	<u>Melt</u>	<u>Anneal/ Extend/Cleave</u>
	15 seconds	60 seconds
20	95°C	60°C

The results are shown in Fig. 5. CE separation of a 1:10 mixture of the 40 cycles products of Alleles 1 and 2. Experimental conditions were as given for the experiments of Fig. 2.

25          Example 5

Electroseparation of Label Conjugates on Microfluidic Chip

Label conjugates comprising fluorescein linked to three different peptides, namely, KKAA, KKKA and KKKK were prepared as follows: The protected tetrapeptide was prepared on resin using Merrifield reagents. The N-terminus of the last aminoacid was

reacted with fluorescein N-hydroxysuccinimide (Molecular Probes). The peptides were cleaved from the resin and purified by high performance liquid chromatography (HPLC).

The label conjugates prepared as described above and fluorescein were combined in an aqueous buffered and were separated and detected in an electrophoresis chip. Detection was 0.5 cm for the injection point on the anodal side of an electrophoresis channel. FITC-KKKK exhibited negative charge and FITC-KKKA and FITC-KKKK exhibited positive charge as determined by the migration time relative to EOF. The net charge of FITC-KKKK was greater than +1 and FITC-KKKA and FITC-KKKK migrated electrophoretically against the EOF. The results are shown in Fig. 6.

10

#### Example 6

##### Capillary Electrophoresis of CFTR PCR products with E-TAG Probes on ABI 310

The following example demonstrates separation in a gel based capillary electrophoresis of cleavage of a probe. The conditions employed were: Gel: 2.5% LDD30 in 1XTBE with 15 7M urea; CE: PE ABI 310; Capillary: 47cm long; 36cm to window; 75um ID; Running Buffer: 1X TBE. (LDD30 is a linear copolymer of N,N-diethyl acrylamide and N,N-dimethylacrylamide, 70:30).

The ABI310 was set up in accordance with the directions of the manufacturer.

The parameters used were: Inj Secs 5; Inj kV 2.0; Run kV 9.4; Run C 45; Run Time 20 min. To determine the relationship of where each probe separated, a spike in system was used. First one digested probe was separated and its peak site determined, then a second probe was spiked into the first probe and the two separated. Then, a third probe was spiked in and separated, and so on till the sites of all the six probes was determined. The single plex PCR runs were first separated followed by separation of the multiplex PCR, which was compared 25 to the S1 digested separation.

Sample*	Type of probe	Probe conc. (nM)	vol (ul)
10s FAM-T	32mer	20	10
10s FAM-T	dig	20	10
10s FAM-T	pcr	80	5

10as HEX-T	32mer	20	10
10as HEX-T	dig	20	10
10as HEX-T	pcr	80	5
11s HEX-A	28mer	20	10
11s HEX-A	dig	20	10
11s HEX-A	pcr	80	5
11as TET-C	28mer	20	10
11as TET-C	dig	20	10
11as TET-C	pcr	80	5
13s FAM-C	23mer	20	10
13s FAM-C	dig	20	10
13s FAM-C	pcr	80	5
13as TET-A	23mer	20	10
13as TET-A	dig	20	10
13as TET-A	pcr	80	5
MP10s11s13as	pcr	80	5
MP10as11as13a	pcr	80	5
MP10s10as11s11as13s13a	pcr	80	5
s	pcr	80	5

dig - S1 nuclease digestion; pcr - amplification; \* the particular samples are found in the above table, where as exemplary 10s FAM-T intends Exon 10 sense, which is referred to as CF7, so that one looks to CF7 for the probe sequence, FAM intends fluorescein, and T is the nucleotide to which the fluorescein is attached. For the other symbols, "as" is the antisense sequence, HEX is hexachlorofluorescein, TET is tetrachlorofluorescein, and when more than one exon is indicated, the reaction mixture is multiplexed under the conditions described below

10

#### Example 7

Taq DNA Polymerase exhibits 5' to 3' exonuclease activity in which hybridized probes on the template DNA are cleaved during PCR. In the subject example, sequence specific probes with fluorescent dye attached to the 5' were employed. PCR was performed with these probes in a reaction and then separation performed in a gel based capillary electrophoresis to determine the cleavage of the probe.

## Primers, Probes, Mutation

Name	Location_SNP	Mutation	SNP
CF1	Exon 11	R553X	C1789T
CF2	Exon 19	R1162X	C3616T
CF4	Exon 3	G85E	G386A
CF5	Exon 4	R117H	G482A
CF6	Exon 7	R347P	G1172C
CF7	Exon 10	V520F	G1690T
CF8	Exon 11	G542X	G1756T
CF9	Exon 11	G551D	G1784A
CF10*	Exon 11	R560T	G1811C
CF11*	Exon 18	D1152H	G3586C
CF13*	Exon 22	G1349D	G4178A
Name	Hyb_probe_length	Probe_seq	Probe_antisense
CF1HYB	26	GTGGAGGTCAACGAGCAAGAATTCT	AGAAATTCTTGCTCGTTGACC TCCAC
CF2HYB	25	AGATGCGATCTGTGAGCCGAGTCCT	AAGACTCGGCTCACAGATCGC ATCT
CF4HYB	32	TTCTGGAGATTTATGTTCTATGGAATC	AAAAAGATTCCATAGAACATA AATCTCCAGAA
CF5HYB	21	AAGGAGGAACGCTCTATCGCG	CGCGATAGAGCGTTCCCTCCTT
CF6HYB	20	ATTGTTCTGCGCATGGCGGT	ACGCCATGCGCAGAACAAAT GCATGCTTGATGACGCTTCT
CF7HYB	25	ATACAGAACGCTCATCAAAGCATGC	GTAT
CF8HYB	29	CAATATAGTTCTGGAGAACGGTGAAT	TGATTCCACCTCTCCAAGAA CTATATTG
CF9HYB	26	CTGAGTGGAGGTCAACGAGCAAGAAT	ATTCTTGCTCGTTGACCTCCAC TCAG
CF10HYB	32	TTCCATTTCCTTTAGAGCACTATACA	TCTTTGTATACTGCTCTAAA AGAAAAATGGAA
CF11HYB	28	AACACTCCAGCATAGATGTGGATAGCTT	CAAGCTATCCACATCTATGCT GGAGTT
CF13HYB	23	CTAACGCATGGCCACAAGCAGTT	AACTGCTTGTGGCCATGGCTT AG
Name	product_size	forward_seq	Reverse_seq
CF1PF/R	198	CCTTTCAAATTCAAGATTGAGCATAAC	TTTACAGCAAATGCTTGCTAG AC
CF2PF/R	127	TGTGAAATTGTCGCCATTCTTA	GGTTTGGTTGACTTGGTAGGT TTA
CF4PF/R	239	TCTTTGCAAGAGAATGGGATAGA	TGGAGTTGGATTCATCCTTAT ATT
CF5PF/R	151	CCAAAGCAGTACAGCCTCTCTTA	CCAAAAATGGCTGGGTGAG
CF6PF/R	137	TCTGTGCTTCCCTATGCACTAA	CCAAGAGAGTCATACCATGTT TGTA
CF7PF/R	146	TGGAGCCTTCAGAGGGTAA	TGCTTGTGACGCTTCTGTA
CF8PF/R	198	CCTTTCAAATTCAAGATTGAGCATAAC	TTTACAGCAAATGCTTGCTAG AC
CF9PF/R	198	CCTTTCAAATTCAAGATTGAGCATAAC	TTTACAGCAAATGCTTGCTAG AC
CF10PF/R	108	GACCAAGGAAATAGAGAGGAAATGTA	CATCTAGGTATCCAAAAGGAG AGTCTA
CF11PF/R	188	GAAGGAGAAGGAAGAGTTGGTATTAT	CGGTATATAGTTCTCCTCATG

*	C	CTATT
CF13PF/R	138	TTGGGCTCAGATCTGTGATAG
*		GCAAGATCTTCGCCCTACTG
Name	Name	Tm_prob, Tm_forward, Tm_reverse, oC forward_length, reverse_length
CF1HYB	CF1PF/R	66.83, 60.36, 58.78 25, 23
CF2HYB	CF2PF/R	68.65, 59.64, 60.51 23, 24
CF4HYB	CF4PF/R	64.24, 60.21, 59.2 23, 25
CF5HYB	CF5PF/R	65.06, 60.08, 60.36 23, 20
CF6HYB	CF6PF/R	68.18, 59.9, 59.48 22, 25

The procedure employed in carrying out the Single-plex PCR reaction was as follows:

1. Make up Master Mix

	1x	6.5x	
5	13.2ul	85.8ul	Water
	3ul	19.5ul	25mM MgCl2
	2.5ul	16.25ul	10x PCR Buffer
	1ul	6.5ul	20ng/ul DNA template
10	0.2ul	1.3ul	25mM dNTPs
	0.3ul	1.95ul	5u/ul Taq Gold (this is added just prior to start of reaction)

2. Aliquot .8ul of 5uM probe and 4ul of 10uM primer set to PCR tubes.

3. Primer sets                  Probe

15	10s	CF10s
	10as	CF10as
	11s	CF11s
	11as	CF11as
	13s	CF13s
	13as	CF13as

20 4. Aliquot 20.2ul of the Master Mix to each tube.

5. In a PE2400 cycler,

96C; 10 MIN

35CYCLES

95C; 10 SEC

25 55C; 30 SEC

70C; 45 SEC

35CYCLES

70C; 10 MIN

4C; 24 hours

5        6. After PCR, run the 2.5ul of each sample on a 2.5% agarose gel.  
7. EtBr stain the gel, take image with camera equipped UV source.

Results clearly demonstrated the formation of a unique electrophoretic tag with distinct mobility (**Table 1**) for each amplified sequence.

10 Multiplex Amplification of CFTR Fragments with E-TAG Probes

In this study the reaction involved a plurality of probes in the same PCR reaction mixture for different snps in CFTR. In the subject system, sequence specific probes with fluorescent dye attached to the 5' terminus of the probe were employed. PCR was performed with these probes and then separation performed in gel based capillary electrophoresis to 15 determine the cleavage of the probe. The following table indicates the fragment, the mutation reference and the specific nucleotide difference and number in the sequence.

The procedure employed for performing the multiplex amplification was as follows:

Make up Master Mix

	<u>1x</u>	<u>2.2x</u>	
20	8ul	17.6ul	25mM MgCl <sub>2</sub>
	2.5ul	5.5ul	10x PCR Buffer
	8ul	17.6ul	10ng/ul DNA template
	.2ul	.44ul	25mM dNTPs
25	1ul	2.2ul	5u/ul Taq Gold (this is added just prior to start of reaction)

8. Aliquot 0.8ul of each 5uM probes CF10s, CF11s, CF10as, CF11as, CF13as and 1ul of each 10uM primer sets 10s, 11s, 10as, 11as, 13as in one PCR tube.  
9. Aliquot 19.7ul of the Master Mix to each tube.  
30        10. In a PE2400 cycler,

96C; 10 MIN  
40CYCLES  
95C; 10 SEC  
55C; 30 SEC  
5 65C; 1 MIN  
40CYCLES  
70C; 10 MIN  
4C; storage

11. After PCR, The amplified products were separated as described in the previous  
10 section. The results are shown in Figure 7. Even in the multiplexed amplification each  
detection probe gives rise to a unique electrophoretic tag with distinct mobility.

#### Example 8

##### Electroseparation of Nine Electrophoretic Tags on Microfluidic Chip

15 Label conjugates comprising 9 different fluorescein derivatives linked to thymine,  
(Table 5; 1-9): Poly deoxy thymidine (20-mer; with a 5' thiol group) is reacted with different  
maleamide functionalized fluoresceins. After the reaction the product is ethanol precipitated.  
In a reaction of 12 1 in volume, 10 1 of 25M oligo, 1.0 1 10X S1 nuclease reaction buffer, 1 1  
20 of S1 nuclease, incubate at 37 °C for 30 min followed by 96 °C for 25 min. The digested  
fragments are purified by HPLC.

The nine different electrophoretic tags prepared as described above and fluorescein  
were combined in an aqueous buffered and were separated and detected in an  
electrophoresis chip. Detection was 0.5 cm for the injection point on the anodal side of an  
25 electrophoresis channel. The results are shown in Fig. 8.

#### Example 9

##### **RT-PCR Conditions:**

Ten ul from a total volume of 25uls of each mRNA was analyzed in a total volume of  
30 50 uls containing 0.5uM of each of the oligonucleotide primers, 0.2mM of each dNTP,

100nM of each E-TAG labeled oligonucleotide probe, 1x RT PCR buffer, 2.5mM MgCl<sub>2</sub>, 0.1U/uL Tfl DNA polymerase and 0.1U/uL AMV Reverse Transcriptase (Promega Access, RT-PCR system).

Reverse Transcription was performed for 45 minutes at 48°C followed by PCR. (40 thermal cycles of 30s at 94°C, 1 min at 60°C and 2 min at 69°C. mRNA was obtained from M.Williams, Genentech Inc. Probe and primer design was performed as described in Analytical Biochemistry, 270, 41-49 (1999). Phosphorothioates were attached to 2, 3,4 and 5 phosphate moieties from the 5' end. Separation was performed as described in the previous section.

**Figure 9a:** Demonstrates the formation of 5 different cleavage products in the PCR amplification of ANF with electrophoretic tag labeled at the 5' end of the sequence detection probe. In the second experiment, phosphate group at 2,3,4 and 5 position is converted into thiophosphate group. PCR amplification of ANF using thiophosphate modified sequence detection probe yield only one cleavage product.

**Figure 9b** Demonstrates the formation of 3 different cleavage products in the PCR amplification of GAPDH with electrophoretic tag labeled at the 5' end of the sequence detection probe. In the second experiment, phosphate group at 2 and 3 position is converted into thiophosphate group. PCR amplification of ANF using thiophosphate modified sequence detection probe yield only one predominant cleavage product.

Results clearly demonstrate that for two different genes that thiophosphates prevent cleavage at multiple sites of detection probes.

A single detectable entity (a single electrophoretic tag: **Figure 9a and 9b**) is generated as a consequence of amplification reaction.

**General Procedure for Synthesis of 6-Carboxyfluorescein Phosphoramidite Derivatives.**

To a solution of 6-carboxyfluorescein (0.5 g, 1.32 mmol) in dry pyridine (5 mL) was added dropwise, isobutyric anhydride (0.55 mL, 3.3mmol). The reaction was allowed to stir at room temperature under an atmosphere of nitrogen for 3 h. After removal of pyridine *in vacuo* the residue was redissolved in ethyl acetate (150 mL) and washed with water (150 mL).

The organic layer was separated, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated *in vacuo* to

yield a brownish residue. This material was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) after which N-hydroxy succinimide (0.23 g, 2.0 mmol) and dicyclohexylcarbodiimide (0.41 g, 1.32 mmol) were added. The reaction was allowed to stir at room temperature for 3 h and then filtered through a fritted funnel to remove the white solid, which had formed. To the filtrate was 5 added aminoethanol (0.12 mL, 2.0 mmol) dissolved in 1 mL of CH<sub>2</sub>Cl<sub>2</sub>. After 3 h the reaction was again filtered to remove a solid which had formed and then diluted with additional CH<sub>2</sub>Cl<sub>2</sub> (50 mL). The solution was washed with water (150 mL) and then separated. The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated *in vacuo* to yield a white foam (0.7 g, 95%, 3 steps). <sup>1</sup>H NMR: (DMSO) 8.68 (t, 1H), 8.21 (d, 1H), 10 8.14 (d, 1H), 7.83 (s, 1H), 7.31 (s, 2H), 6.95 (s, 4H), 4.69 (t, 1H), 3.45 (q, 2H), 3.25 (q, 2H), 2.84 (h, 2H), 1.25 (d, 12 H). Mass (LR FAB<sup>+</sup>) calculated for C<sub>31</sub>H<sub>29</sub>NO<sub>9</sub> (M+H<sup>+</sup>) 559.2, found: 560.

All publications and patent applications cited in this specification are herein 15 incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent 20 to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

**WHAT IS CLAIMED IS:**

1. A method for detecting at least one nucleic acid sequence in a target nucleic acid sample, said method comprising:

5 combining under primer extension conditions: a polymerase having 5'-3' exonuclease activity, said target DNA and a reagent pair consisting of a primer and detection sequence comprised of nucleotide bases for each DNA sequence to be determined, wherein each said primer specifically binds to said target DNA and said detection sequence binds to said target DNA downstream from said primer in the direction  
10 of primer extension, wherein each said detection sequence is characterized by having a electrophoretic tag specific for each said DNA sequence;

executing at least one cycle of said primer extension, whereby said detection sequence bound to target DNA is at least partially degraded with release of said electrophoretic tag substantially free of said detection sequence;

15 separating released electrophoretic tags into individual fractions; and detecting, by means of said tag, said fractions, whereby the presence in said target DNA sample of said at least one DNA sequence is detected;

20 with the proviso that, when separation is performed solely by means of differences in mass, the electrophoretic tags that are separated all have the same number of nucleotides bonded to the electrophoretic tag.

25 2. A method according to Claim 1, wherein a plurality of said pairs are combined for determining a plurality of single nucleotide polymorphisms ("snps") in said target DNA, wherein said detection sequences bind at the site of said snps.

3. A method according to Claim 1, wherein said executing comprises a plurality of cycles.

4. A method according to Claim 1, comprising the additional step of reacting said electrophoretic tag with a fluorophore after release of said electrophoretic tag.
5. A method according to Claim 1, wherein said electrophoretic tag comprises a neutral linker of from 1 to 60 carbon atoms.
6. A method according to Claim 1, wherein said electrophoretic tag comprises a charged linker of from 1 to 60 carbon atoms.
- 10 7. A method according to Claim 1, wherein said detection sequence comprises a linkage resistant to exonuclease hydrolysis between at least the second and third bases and optionally successive bases in the direction of degradation.
- 15 8. A method for detecting a plurality of single nucleotide polymorphisms ("snps") in a target DNA sample, said method comprising:
  - combining under primer extension conditions: a polymerase having 5'-3' exonuclease activity; said target DNA and a reagent pair consisting of primer and snp detection sequence for each snp, wherein said primer specifically binds to said target DNA and said snp detection sequence binds to said target DNA at the site of said snp downstream from said primer in the direction of primer extension, wherein said snp detection sequence is characterized by having an electrophoretic tag with a detectable label;
  - executing a plurality of cycles of said primer extension, whereby said snp detection sequences bound to target DNA are partially degraded with release of said electrophoretic tag substantially free of said detection sequence ;
  - 25 separating said released electrophoretic tags into separate bands by means of capillary electrophoresis; and
  - detecting said bands of electrophoretic tags,
  - whereby snps in said sample DNA are detected.

9. A method according to Claim 8, wherein said pair have a total of at least 36 nucleotides.

10. A method according to Claim 8, wherein said snp detection sequence is further characterized by comprising a quencher, which quenches said fluorophor when bound to said snp detection sequence, and including the further step of monitoring the change in fluorescence during said executing.

11. A method according to Claim 8, wherein said snp detection sequences are within 10 200 nucleotides of said primer when bound to said target DNA.

12. A method according to Claim 8, wherein said snp detection sequence comprises a positively charged moiety.

15 13. A method according to Claim 12, wherein said charged moiety comprises at least one of lysine, arginine and histidine.

14. A method for determining the amount of at least one single nucleotide polymorphism ("snp") in a target DNA sample, said method comprising:

20 combining under primer extension conditions: a polymerase having 5'-3' exonuclease activity, said target DNA, a reagent pair consisting of a primer and snp detection sequence for each snp to be determined, wherein each said primer specifically binds to said target DNA and said snp detection sequence binds to said target DNA downstream from said primer in the direction of primer extension and at the site of said 25 snp, wherein each said snp detection sequence is characterized by having a labeled electrophoretic tag specific for a snp, and a quantitating system comprising at least two control sequences having a common primer region and different control detection regions downstream from said primer region in the direction of primer extension, a primer sequence complementary to said primer region and a control detection sequence for each 30 of said control detection regions characterized by having a labeled electrophoretic tag

specific for the control detection sequence to which it is bound, said two control sequences differing in concentration;

executing at least one cycle of said primer extension, whereby said snp detection sequence bound to target DNA and said control detection sequences are at least partially degraded with release of said electrophoretic tags substantially free of said detection sequence;

electrophoretically separating said electrophoretic tags into separate bands;  
determining the signal from said label from each of said electrophoretic bands; and  
comparing the band signals from said electrophoretic tags from said control  
detection sequences with the signals from said snp detection sequences;  
whereby the amount of a snp in said sample is determined.

15. A method according to Claim 14 wherein at least 3 different control sequences are employed with their respective control detection sequences, and the concentration of each of said control sequences differs by at least a factor of 100 from another control sequence.

16. A method according to Claim 14, wherein said control sequences are of at least about 100 nucleotides.

20 17. A method according to Claim 14, wherein said control sequences comprise a linkage resistant to exonuclease hydrolysis between the second and third bases in the direction of degradation

25 18. A kit comprising a plurality of snp detection sequences characterized by:  
consisting of at least 12 nucleotides;

the 5'-nucleotide bonded to an electrophoretic tag;

the penultimate nucleotide bonded to the adjacent nucleotide by a link resistant to exonuclease hydrolysis; and

a complementary nucleotide to a snp at other than the terminal nucleotide.

19. A kit according to Claim 18, wherein said electrophoretic tag comprises a fluorophor.

20. A kit according to Claim 18, wherein said electrophoretic tag comprises an amino acid or peptide.  
5

21. A kit according to Claim 20, wherein said amino acid or said peptide comprises lysine, arginine or histidine.

10 22. A kit comprising a plurality of compounds of the formula:

R - L - T:

wherein R is a fluorescer;

L is a linking group selected from the group consisting of NH-lysine, NH-(lysine)<sub>2</sub>, NH-alanine, NH-aspartic acid, NH-(aspartic acid)<sub>2</sub>, NH-(aspartic acid)<sub>3</sub>, NH-(aspartic acid)<sub>4</sub>,  
15 NH-(aspartic acid)<sub>5</sub>, NH-(aspartic acid)<sub>6</sub>, NH-(aspartic acid)<sub>7</sub>, NH-alanine-lysine, NH-aspartic acid-lysine, NH-(aspartic acid)<sub>2</sub>-lysine, NH-(aspartic acid)<sub>3</sub>-lysine, NH-(aspartic acid)<sub>4</sub>-lysine, NH-(aspartic acid)<sub>5</sub>-lysine, NH-(aspartic acid)<sub>6</sub>-lysine, NH-(aspartic acid)<sub>7</sub>-lysine, NH-(aspartic acid)<sub>8</sub>-lysine, NH-(lysine)<sub>4</sub>, and NH-(lysine)<sub>5</sub>; and

20 T is selected from the group consisting of purine, pyrimidine, nucleoside, nucleotide, and nucleotide triphosphate.

23. In a method according to Claim 1, where the E-TAGs all have the same number of nucleotides bound to said E-TAG, the improvement which comprises:

25 providing a mixture of E-TAGs substantially having the same number of nucleotides bound to the E-TAG by:

the E-TAGs having the penultimate linkage and optionally successive linkages of said detection sequence nuclease resistant to hydrolysis;

said E-TAG is labeled at the penultimate or terminal nucleotide with a label that binds to a positively charged receptor, and including the additional step of combining said released

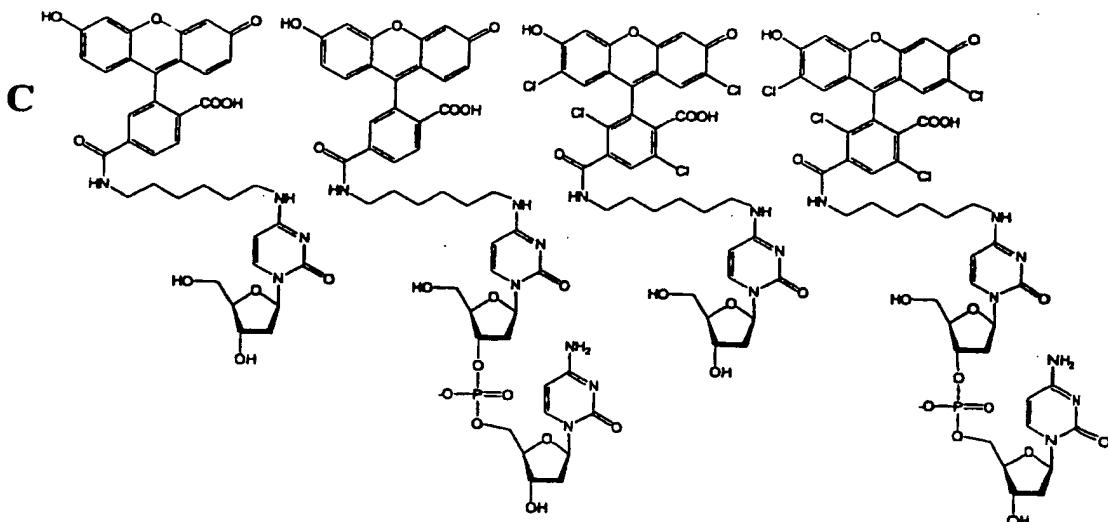
E-TAGs with said positively charged receptor and separating said E-TAGs into fractions by electrophoresis; or

5 said E-TAG is labeled at the penultimate nucleotide or terminal nucleotide with a label that binds to a receptor, and including the additional step of combining said released E-TAGs with said receptor, and including the additional step of separating E-TAGs having the same number of nucleotides by means of said receptor.

### Fluorescent Dyes

	<u>Absorbance Maxima</u>	<u>Emission Maxima</u>
Fluorescein	494 nm	525 nm
B Tetrachloro Fluorescein	521 nm	536 nm
TAMRA	565 nm	580 nm

Cleaved Fragments:



A

CCA GCA ACC AAT GAT GCC CGT T-TAMARA-3'  
CA GCA ACC ATT GAT GCC CGT T-TAMARA-3'

CCA GCA AGC ACT GAT GCC TGT T-TAMARA-3'  
CA GCA AGC ACT GAT GCC TGT T-TAMARA-3'

Fig 1

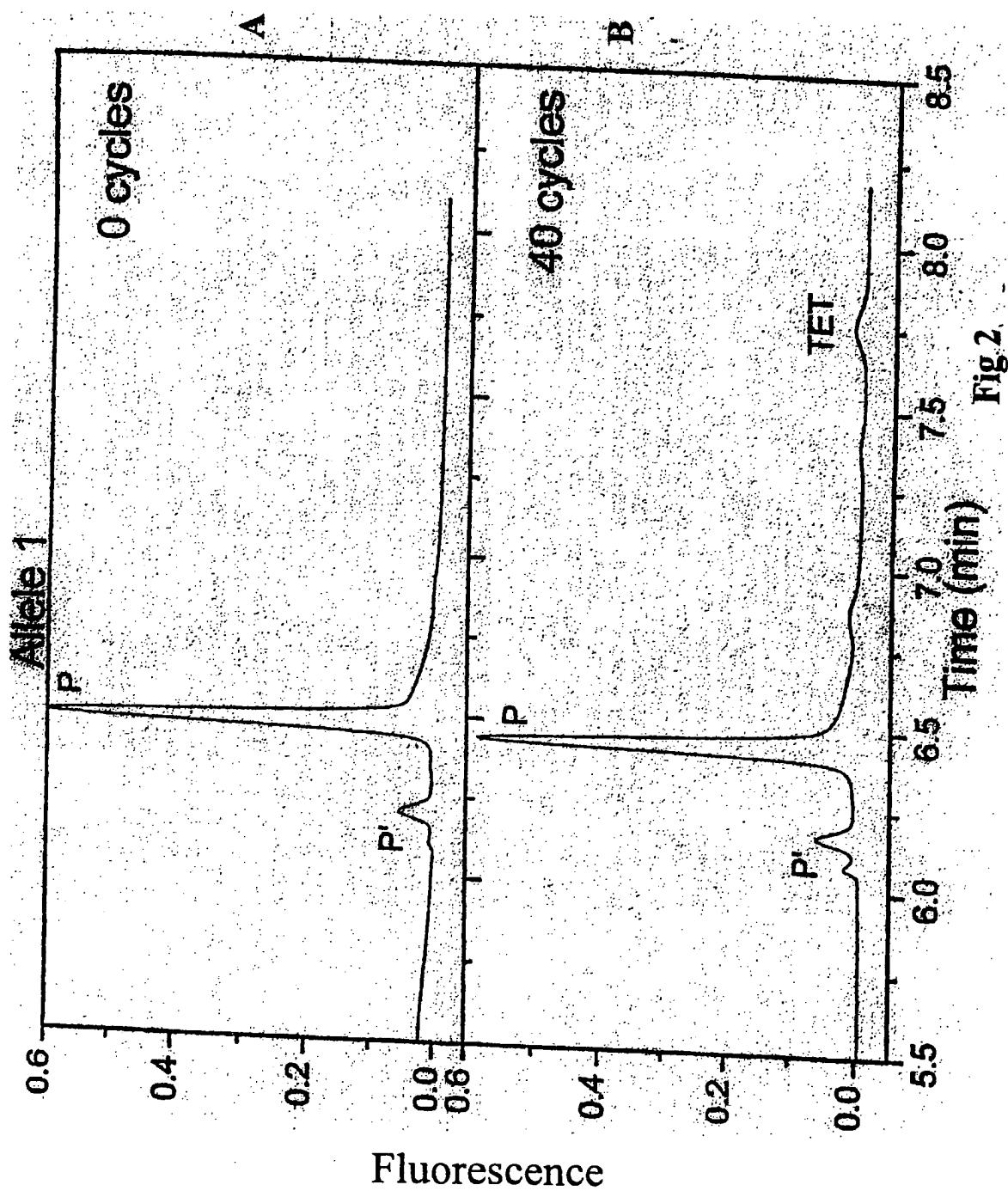


Fig 2

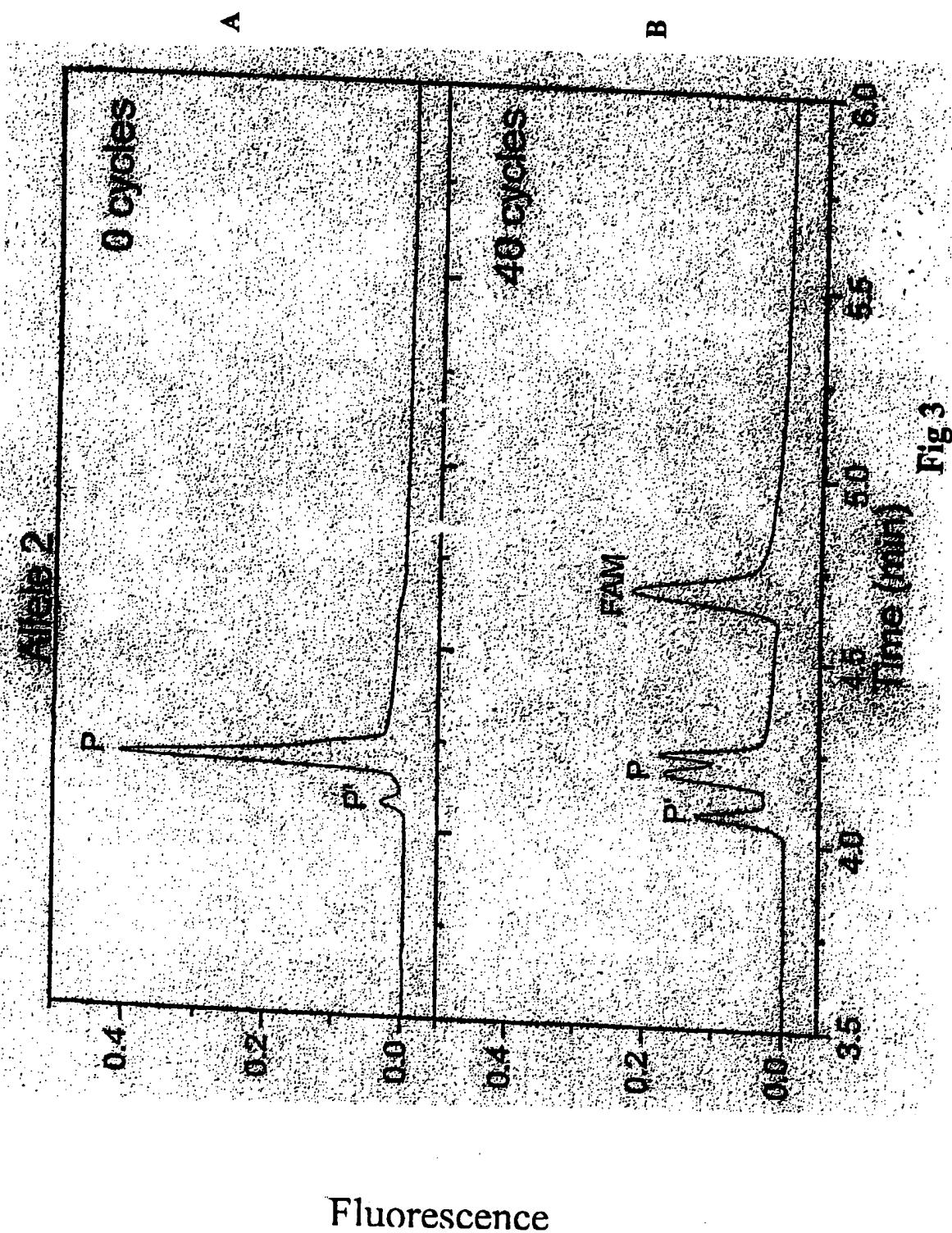


Fig. 3

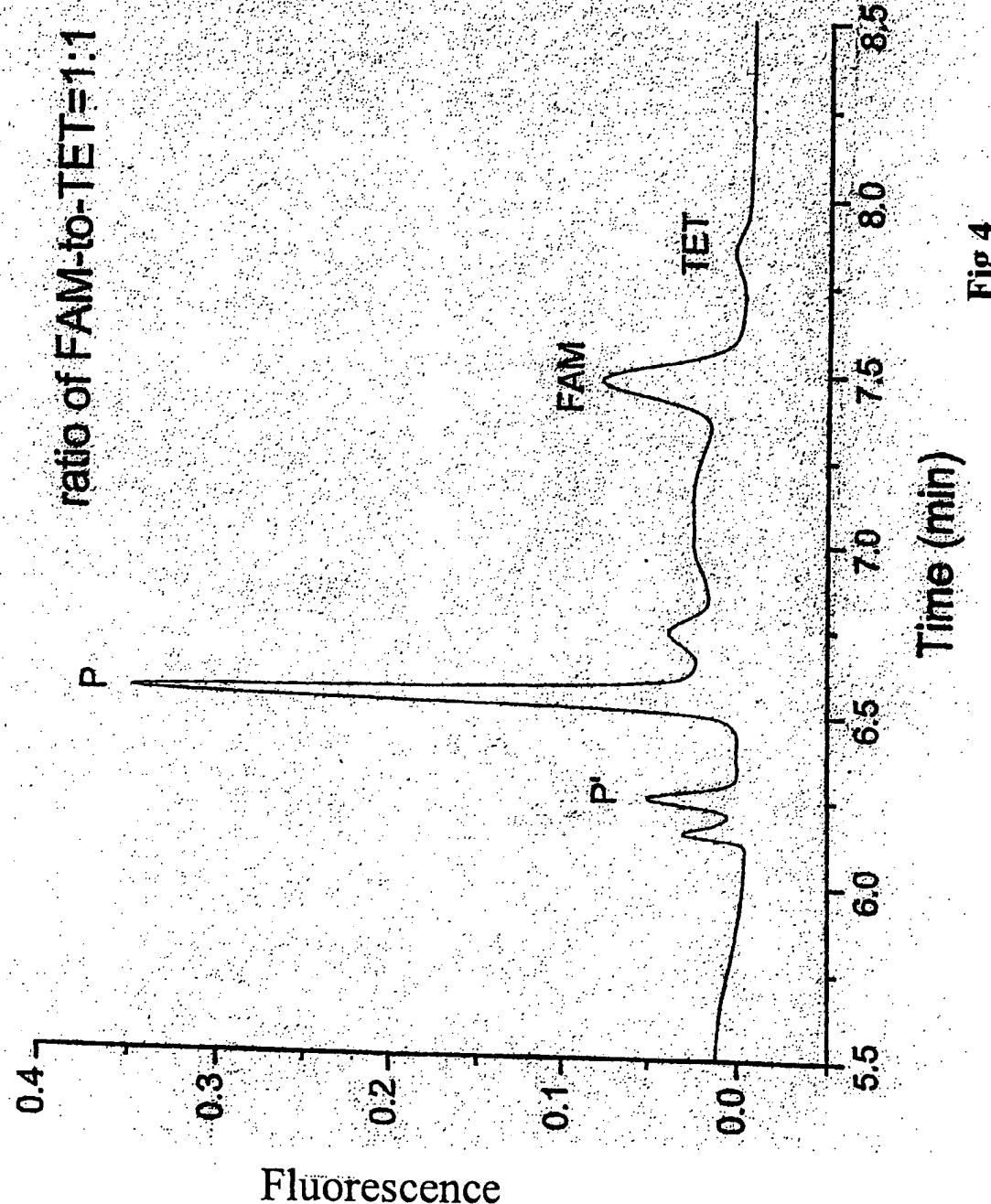


Fig 4

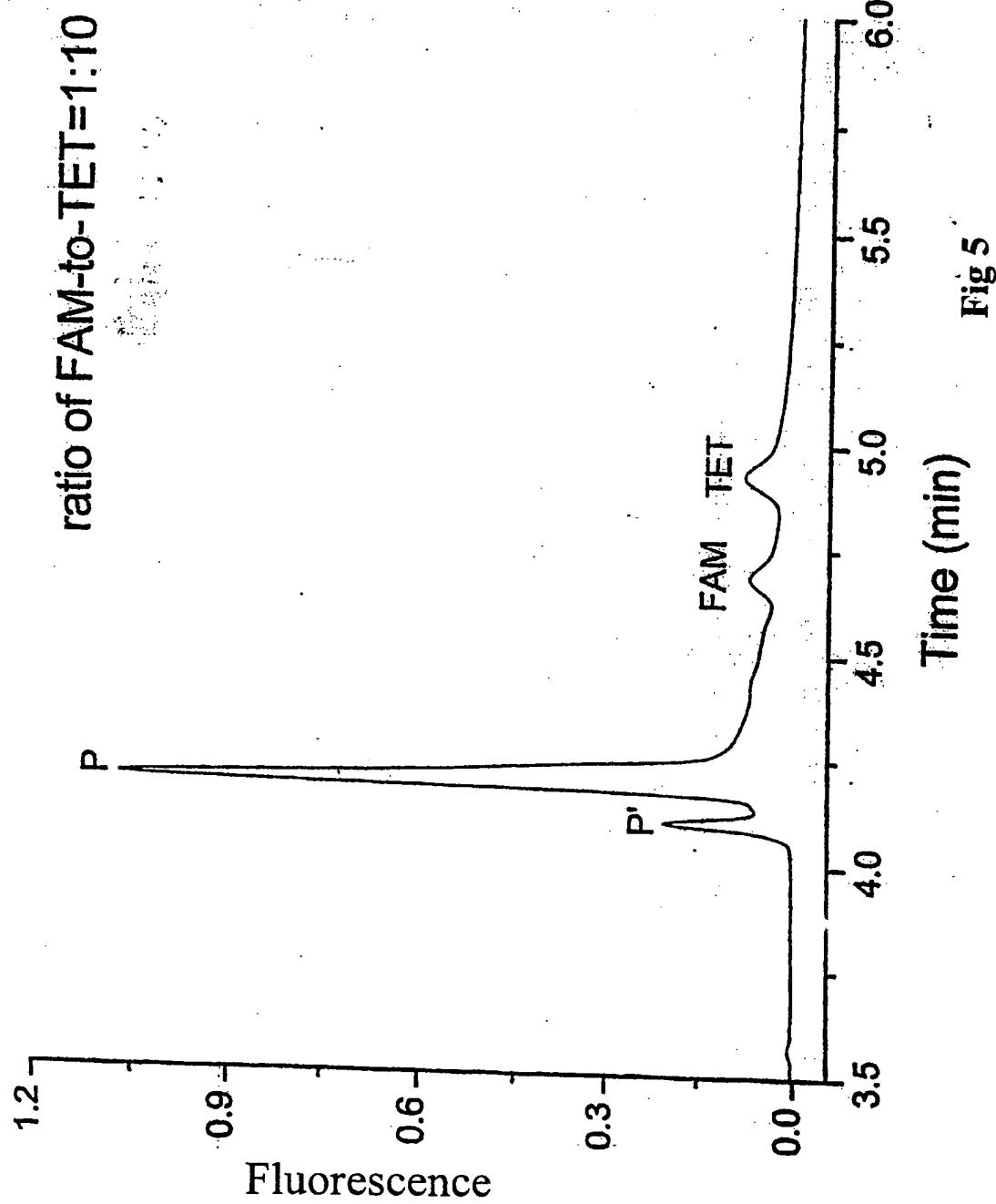


Fig 5

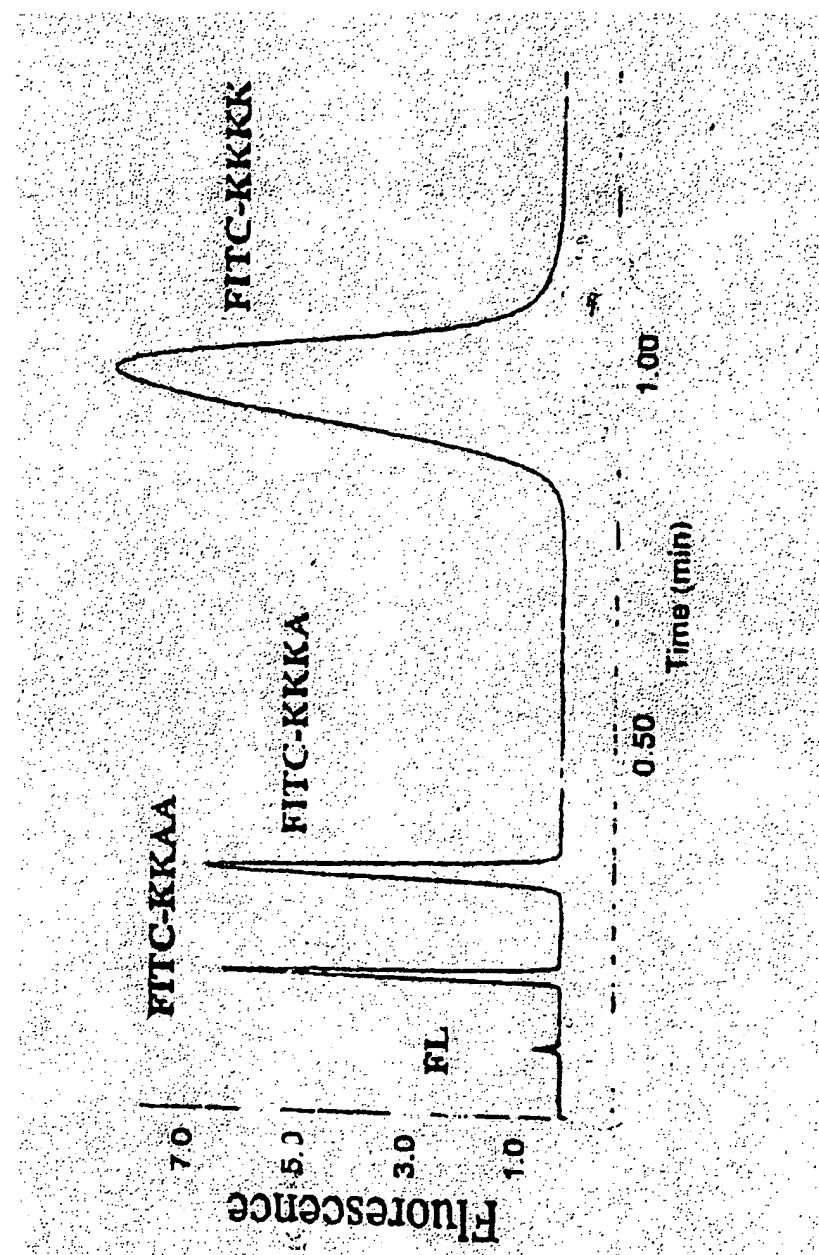
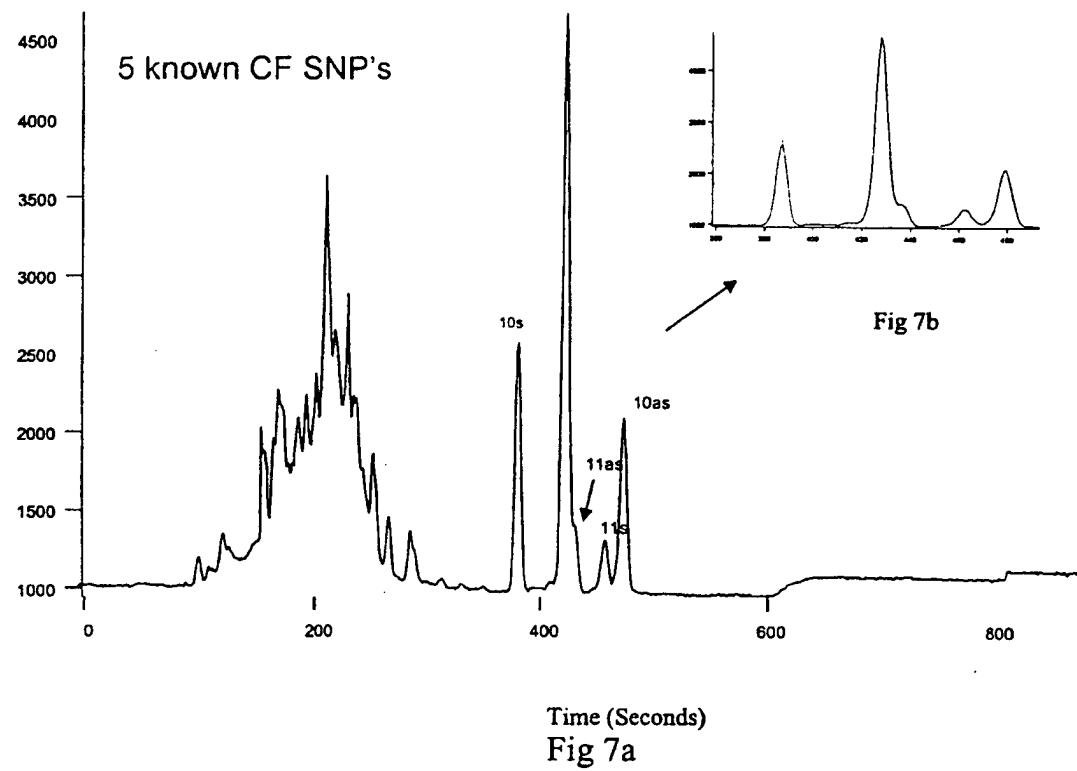
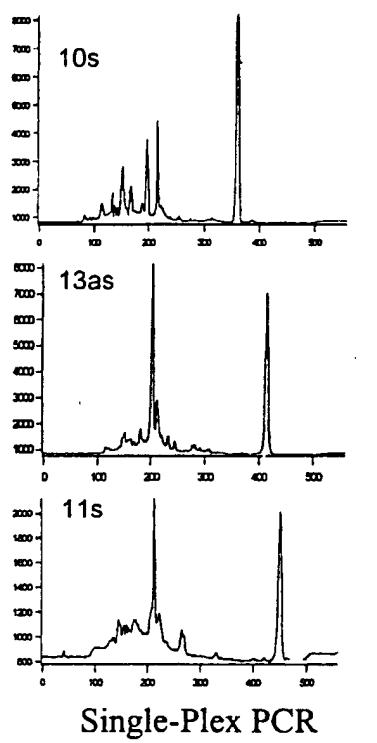


Fig 6

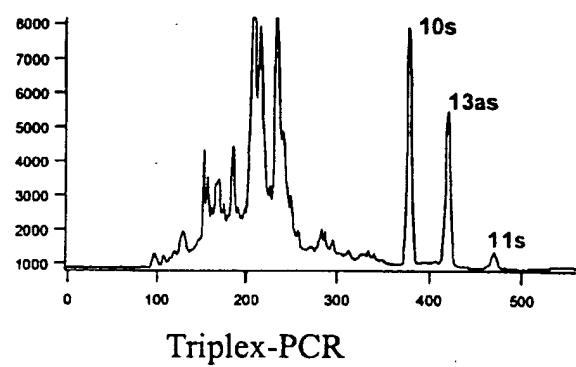
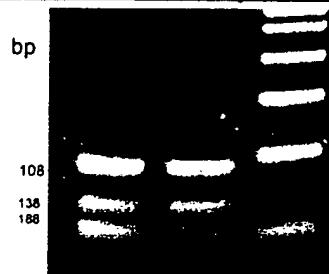


Time (Seconds)  
Fig 7a

(Fig 7b shows an expanded electrophoretic tag region from 380-500 seconds)



Single-Plex PCR

2% Agarose Gel of Triplex PCR

Triplex-PCR

Fig 7c

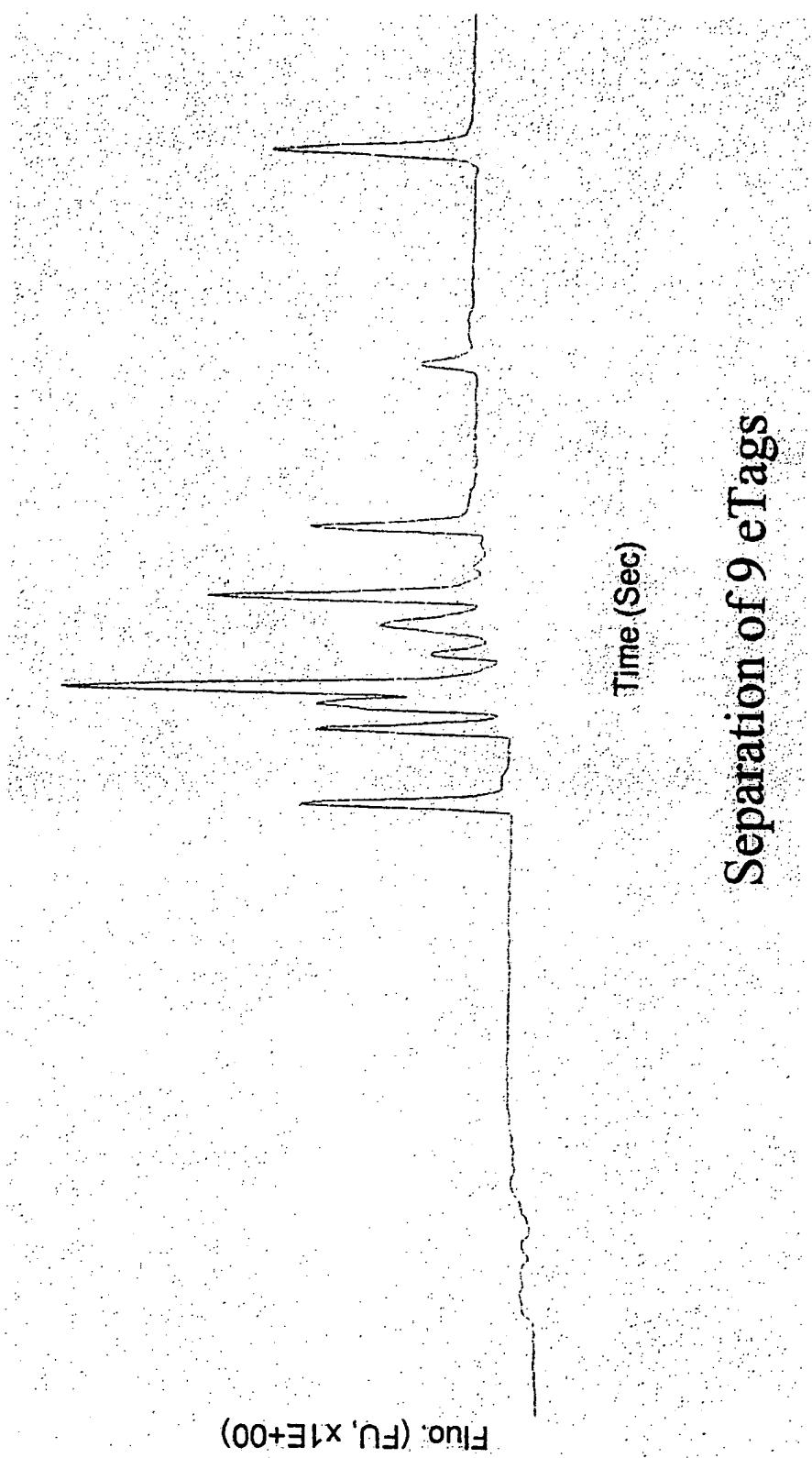


Fig 8

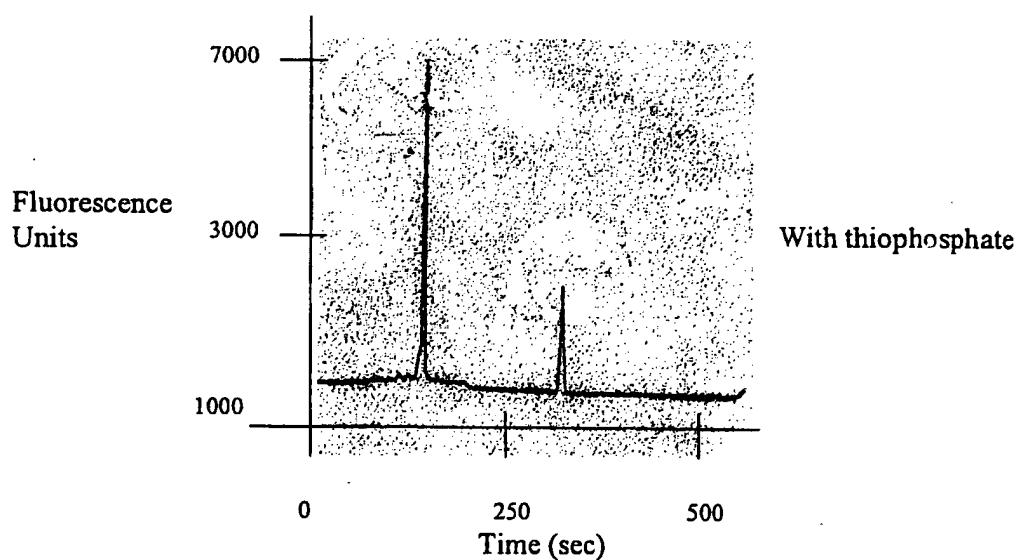
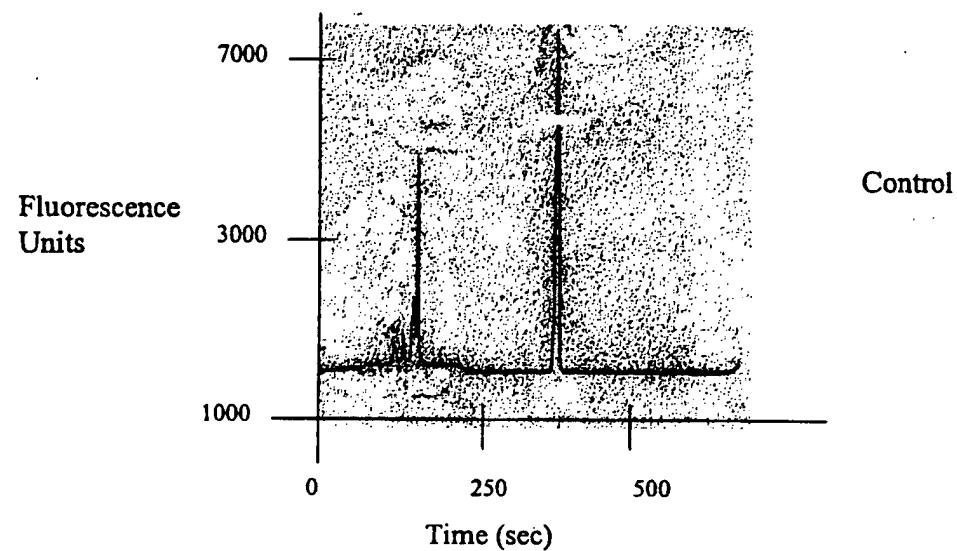
**Effect of Thiophosphate on 5'-3' cleavage: ANF**

Fig. 9A

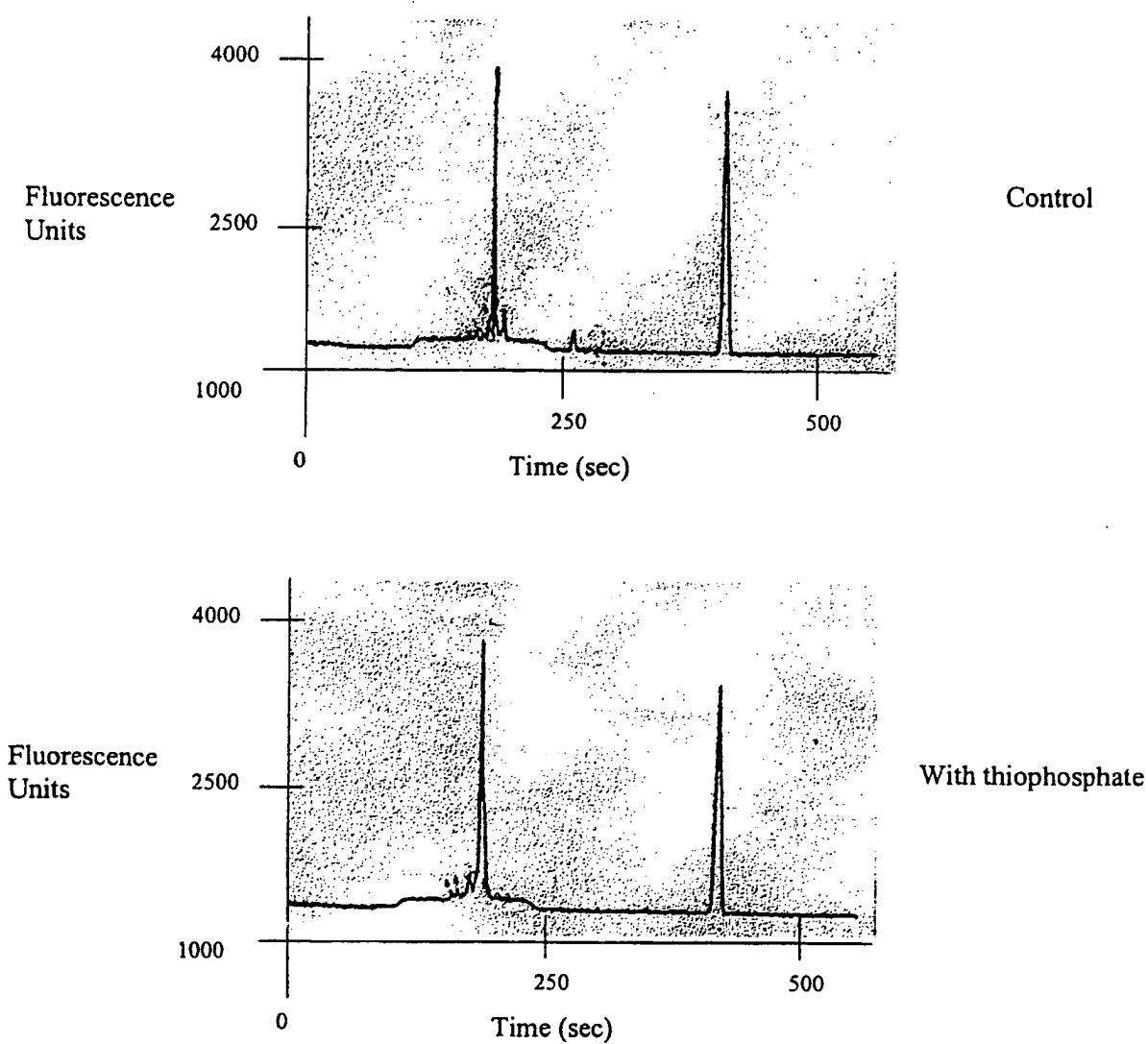
**Effect of Thiophosphate on 5'-3' cleavage: ANF**

Fig. 9B

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/11396

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07H 21/02, 21/04, 21/00; C12Q 1/68, 19/34; C25B 1/00

US CL : 536/23.1, 24.3, 25.32; 435/6, 91.2; 204/182.8

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 24.3, 25.32; 435/6, 91.2; 204/182.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN search, uspat, biosis, medline, caplus  
search terms: single nucleotide polymorphisms, peptide probe, fluorophor

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEE et al. Allelic Discrimination by Nick-Translation PCR with Fluorogenic Probes. Nucleic acid research. 1993, Vol. 21, No. 16, pages 3761-3766, especially page 3761-3762.	8
Y	MARINO et al. Characterization of Mitochondrial DNA Using Low-Stringency Single Specific Primer Amplification Analyzed by Laser Induced Fluorescence-Capillary Electrophoresis. Electrophoresis. 1996, Vol. 17, pages 1499-1504, especially pages 1499-1501.	1 and 9-22

 Further documents are listed in the continuation of Box C. See patent family annex.

- \* Special categories of cited documents:
- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*B\* earlier document published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed
- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*a\* document member of the same patent family

Date of the actual completion of the international search

27 JUNE 2000

Date of mailing of the international search report

11 AUG 2000

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/11396

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOLLAND et al. Detection of Specific Polymerase Chain Reaction Product by Utilizing the 5'→3' Exonuclease Activity of <i>Thermus aquaticus</i> DNA polymerase. Proc. Natl. Acad. Sci., USA. August 1991, Vol. 88, pages 7276-7280, see entire document.	1 and 9-22
Y	US 5,874,213 A (CUMMINS et al.) 23 February 1999, the Abstract, column 11, lines 48-54, column 13, lines 24-49.	1 and 9-22